(51) International Patent Classification 5 :		(11) International Publication Number: WO 91/19810
C12P 21/00, A01H 5/00	A1	(43) International Publication Date: 26 December 1991 (26.12.91)

(21) International Application Number: PCT/US91/04447

(22) International Filing Date: 17 June 1991 (17.06.91)

(30) Priority data:
538,857   15 June 1990 (15.06.90)   US
Not furnished   17 June 1991 (17.06.91)   US

(71) Applicant: CALIFORNIA BIOTECHNOLOGY INC. [US/US]; 2450 Bayshore Parkway, Mountain View, CA 94043 (US).

(72) Inventor: CORDELL, Barbara ; 4051 Ben Lomond Drive, Palo Alto, CA 94306 (US).

(74) Agent: BOZICEVIC, Karl; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94043 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

81

(54) Title: TRANSGENIC NON-HUMAN MAMMAL DISPLAYING THE AMYLOID-FORMING PATHOLOGY OF ALZHEIMER'S DISEASE

(57) Abstract

Cloned recombinant or synthetic DNA sequences related to the pathology of Alzheimer's disease are injected into fertilized mammalian eggs (preferably mice eggs). The injected eggs are implanted in pseudo pregnant females and are grown to term to provide transgenic mice whose cells express proteins related to the pathology of Alzheimer's disease. The injected sequences are constructed having promoter sequences connected so as to express the desired protein in specific tissues of the transgenic mammal (most notably in nerve tissue). The proteins which are preferably ubiquitously expressed include (1) β-amyloid core precursor proteins; and (2) β-amyloid related precursor proteins; and (3) serine protease inhibitor. The transgenic mice provide useful models for studying compounds being tested for their usefulness in treating Alzheimer's disease, and for studying the *in vivo* interrelationships of these proteins to each other.

TRANSGENIC NON-HUMAN MAMMAL DISPLAYING THE
AMYLOID-FORMING PATHOLOGY OF ALZHEIMER'S DISEASE

Cross-Reference

This application is a continuation-in-part of
my earlier filed pending U.S. application Serial No.
07/538,857, filed June 15, 1990, which application is
incorporated herein by reference in its entirety and to
which application I claim priority under 35 U.S.C. § 120.

1. Field of the Invention

The invention relates generally to animal
models useful in testing a hypothesis related to the
treatment of a disease. More specifically, it relates to
transgenic mammals which have had incorporated in their
genome specific segments of exogenous genetic material
which encode for and in specific cell types will
ubiquitously over-express b-amyloid proteins, their
precursors and portions of such proteins and precursors.

2. Background of the Invention

The background of the present invention is
twofold in that it relates to: (1) biological organisms
which have been genetically transformed; and (2) a study
of genetic material related to amyloidosis.
Both of these areas are discussed below.

2.1. Genetic Transformations.

For sometime it has been known that it is
possible to carry out the genetic transformation of a

zygote (and the embryo and mature organism which result
therefrom) by the placing or insertion of exogenous
genetic material into the nucleus of the zygote or to any
nucleic genetic material which ultimately forms a part of

5   the nucleus of the zygote. The genotype of the zygote
and the organism which results from a zygote will include
the genotype of the exogenous genetic material.
Additionally, the inclusion of exogenous genetic material
in the zygote will result in a phenotype expression of

10   the exogenous genetic material.

          The genotype of the exogenous genetic material
is expressed upon the cellular division of the zygote.
However, the phenotype expression, e.g., the production
of a protein product or products of the exogenous genetic

15   material, or alterations of the zygote's or organism's
natural phenotype, will occur at that point of the
zygote's or organism's development during which the
particular exogenous genetic material is active.
Alterations of the expression of the phenotype include an

20   enhancement or diminution in the expression of a
phenotype or an alteration in the promotion and/or
control of a phenotype, including the addition of a new
promoter and/or controller or supplementation of an
existing promoter and/or controller of the phenotype.

25   The genetic transformation of various types of
organisms is disclosed and described in detail in U.S.
patent 4,873,191, issued October 10, 1989, which is
incorporated herein by reference to disclose methods of
producing transgenic organisms. The genetic

30   transformation of organisms can be used as an _in vivo_
analysis of gene expression during differentiation and in
the elimination or diminution of genetic diseases.

          The genetic transformation of a zygote (and the
organisms which matures therefrom) is carried out by the

35   addition of exogenous genetic material in a manner such

that the exogenous genetic material becomes part of the
nucleic portion of the zygote prior to a division of the
zygote. If the exogenous genetic material is added after
mitosis or cell division of the zygote, the exogenous
5   genetic material must be added to each resulting nucleus.
However, there is a possibility that the exogenous
genetic material may not be integrated into and become a
part of the genetic material of the zygote and the
organism which results therefrom. Thus, the exogenous
10   genetic material can be added to any nucleic genetic
material which ultimately forms a part of the nucleus of
the zygote, including the zygote nucleus.

The nucleic genetic material of the organism
being transformed must be in a physical state which
15   enables it to take up the exogenous genetic material.
There are numerous ways of accomplishing this. For
example, the exogenous genetic material can be placed in
the nucleus of a primordial germ cell which is diploid,
e.g., a spermatogonium or oogonium. The primordial germ
20   cell is then allowed to mature to a gamete, which is then
united with another gamete or source of a haploid set of
chromosomes to form a zygote.

The exogenous genetic material can be placed in
the nucleus of a mature egg. It is preferred that the
25   egg be in a fertilized or activated (by parthenogenesis)
state. After the addition of the exogenous genetic
material, a complementary haploid set of chromosomes
(e.g., a sperm cell or polar body) is added to enable the
formation of a zygote. The zygote is allowed to develop
30   into an organism such as by implanting it in a
pseudopregnant female. The resulting organism is
analyzed for the integration of the exogenous genetic
material. If positive integration is determined, the
organism can be used for the in vivo analysis of the gene

35

expression, which expression is believed to be related to a particular genetic disease.

Attempts have been made to study a number of different types of genetic diseases utilizing such
5   transgenic animals. Attempts related to studying Alzheimer's disease are disclosed within published PCT application WO89/06689 and PCT application WO89/06693, both published on July 27, 1989, which published applications are incorporated herein by reference to
10   disclose genetic sequences coding for Alzheimer's β-amyloid protein and the incorporation of such sequences into the genome of transgenic animals.

As described in detail below, the production of β-amyloid protein is believed to be related to
15   Alzheimer's disease. However, a serious obstacle to elucidating the molecular mechanism involved in amyloid synthesis and deposition in an Alzheimer's diseased brain has been the unavailability of convincing animal models for this uniquely human disorder. Published PCT
20   applications WO89/06689 and WO89/06693 disclose particular DNA sequences believed to be related to the production of amyloid. These particular sequences are fused to newly developed tumor virus vectors, derived from SV40 and the JC virus to produce constructs. These
25   constructs are utilized to transfect cells and transgenic mice to establish models for amyloid overexpression, which may be related to amyloid accumulation in the Alzheimer's-diseased brain.

The transgenic animals produced in accordance
30   with the present invention are intended to provide an experimental medium for elucidating aspects of the molecular pathogenesis of Alzheimer's disease and to serve as tools for screening drugs that may have potential application as therapeutic agents to prevent or
35   limit amyloid accumulation.

2.2. Alzheimer's Disease and β-Amyloid Protein

It is estimated that over 5% of the U.S. population over 65 and over 15% of the U.S. population over 85 are beset with some form of Alzheimer's disease (Cross, A.J., Eur J Pharmacol (1982) 82:77-80; Terry, R.D., et al., Ann Neurol (1983) 14:497506). It is believed that the principal cause for confinement of the elderly in long term care facilities is due to this disease, and approximately 65% of those dying in skilled nursing facilities suffer from it.

Certain facts about the biochemical and metabolic phenomena associated with the presence of Alzheimer's disease are known. Two morphological and histopathological changes noted in Alzheimer's disease brains are neurofibrillary tangles (NFT) and amyloid deposits. Intraneuronal neurofibrillary tangles are present in other degenerative diseases as well, but the presence of amyloid deposits both in the interneuronal spaces (neuritic plaques) and in the surrounding microvasculature (vascular plaques) seems to be characteristic of Alzheimer's. Of these, the neuritic plaques seem to be the most prevalent (Price, D.L., et al., Drug Development Research (1985) 5:59-68). Plaques are also seen in the brains of aged Down's Syndrome patients who develop Alzheimer's disease.

The protein which makes up the bulk of these plaques has been partially purified and sequenced. Plaque-rich brains of deceased Alzheimer's patients have been used as a source to extract an approximately 4.2 kd "core" polypeptide, amyloid plaque core protein (APCP), herein referred to as "β-amyloid core protein." This peptide was designated β-protein by Glenner, G., et al., [Biochem Biophys Res Commun (1984) 120:885-890]. The amino acid sequence of the amino-terminus has been

determined [Glenner, G., et al., _Biochem Biophys Res
Commun_ (1984) _122_:1131-1135; Masters, C.L., et al., _Proc
Natl Acad Sci USA_ (1985)_82_:4245-4259] and the amino acid
sequences reported by the two groups are identical except
that Glenner et al., report a glutamine at position 11
for Alzheimer Disease cerebral vascular amyloid whereas
Masters et al., report glutamic acid at position 11.
Also, the former authors report that the cerebral
vascular amyloid has a unique amino-terminus while the
latter authors report that the form found in amyloid
plaque cores has a "ragged" amino-terminus - i.e.,
peptides isolated from this source appear to be missing
3, 7, or 8 amino acids from the amino-terminus. Both
groups have shown that the same peptide is found in the
amyloid plaque cores and vascular amyloid of adult Down's
syndrome-afflicted individuals and report glutamic acid
at position 11.

Further studies on the β-amyloid core protein
were also conducted by Roher, A., et al., _Proc Natl Acad
Sci USA_ (1986) _83_:2662-2666 which showed the complete
amino acid composition of the β-protein, and verified
that it matched that of no known protein. The
compositions obtained were, however, evidently not in
agreement with those of Allsop, D., et al., _Brain Res_
(1983) _259_:348352; nor were they in agreement with those
published by Glenner or Masters (supra).

Wong, C.W., et al., _Proc Natl Acad Sci USA_
(1985) _82_:8729-8732 showed that a synthetic peptide which
was homologous to the first ten amino acids of the β-
amyloid core protein described by Masters (supra) was
able to raise antibodies in mice and that these
antibodies could be used to stain not only amyloid-laden
cerebral vessels, but neuritic plaques as well. These
results were confirmed by Allsop, D. et al., _Neuroscience
Letters_ (1986) _68_:252-256 using monoclonal antibodies

directed against a synthetic peptide corresponding to
amino acids 8-17. Thus, in general, the plaque protein
found in various locations of the brain of Alzheimer's
patients appears to be similar in immunoreactivity. It

5   is highly insoluble, as shown by the inability to achieve
solubilization in many commonly used denaturants such as
detergents and chaotropic agents (Masters, supra, Allsop,
D., et al., (supra)).

It is believed, by analogy to some other

10   amyloid proteins, that β-amyloid core protein may be
formed from a precursor in the peripheral circulatory
system or lymphatic system. There are six known
instances of disease-associated amyloid deposits in which
the nature of the precursor protein for the amyloid

15   protein is known: for primary amyloidosis, the source is
an immunoglobulin light chain; for secondary amyloidosis,
the precursor is amyloid A protein; for familial amyloid
polyneuropathy and senile cardiac amyloidosis, prealbumin
also known as transthyreitin or a variant thereof; for

20   medullary carcinoma of thyroid, a procalcitonin fragment;
and for hereditary cerebral hemorrhage, gamma-trace
fragment which has been shown to be cystatin C. (See,
e.g., Glenner, G. New England Journal of Medicine (1980)
302:1283; Sletton, K., et al., Biochem J (1981) 195:561;

25   Benditt, et al., FEBS Lett (1971) 19:169; Sletton, K., et
al., Eur J Biochem (1974) 41:117; Sletton, K., et al., J
Exp Med (1976) 143:993). The foregoing is a partial list
and there are at least a number of additional references
with regard to procalcitonin fragment as a precursor for

30   the amyloid of the thyroid carcinoma. Alternatively, or
additionally, such a precursor for β-amyloid core protein
may be produced in the brain or elsewhere and is
specifically deposited in the brain.

It has been described that a protein containing

35   the β-amyloid core protein (referred to as A4) sequence

-8-

within the framework of a larger protein exists (Kang,
J., et al., Nature (1987) 325:733-736). This protein,
which is a potential precursor in vivo to the β-amyloid
core protein, was predicted from the sequence of a cDNA

5   clone isolated from a human fetal brain tissue cDNA
library and consists of 695 amino acid residues (referred
to as A695) wherein the amino terminus of the β-amyloid
core protein begins at position 597. By analogy to the
above described series, it may be that such a precursor

10   or a fragment thereof circulates in the serum at a higher
level differentiable in Alzheimer's victims relative to
unafflicted individuals. Alternatively or additionally,
such differences may be detected in the cerebral spinal
fluid.

15   It appears as though there are a number of
precursor proteins in addition to A695, which was
described by Kang et al. One such precursor protein is
described in copending U.S. application Serial
No. 361,912, filed June 6, 1989, by researchers from the

20   same research organization as the present inventors
(A751). Others have characterized an additional amyloid
precursor protein (see Kitaguchi et al., Nature 331:530-
532 (1988), which is slightly larger, 770 amino acids.
It is pointed out that these A751 and A770 proteins

25   contain an approximately 57 amino acid insert beyond
A695. This particular 57 amino acid insert sequence is
highly homologous to a number of Kunitz-type inhibitors
which are specific for a number of serine proteases. An
additional 19 amino acids are present adjacent to the 57

30   amino acid insert in the A770 form.
             As indicated by the above publications and
numerous other publications not cited, the genetic
material encoding for the production of β-amyloid
precursor proteins are the subject of intensive study.

35   However, at present, there is no direct verifiable

information available on the specific mechanisms that
regulate the production and deposit of amyloid protein in
an Alzheimer's diseased brain. It is known that the
genetic material encoding for the production of β-amyloid

5   precursor protein is on chromosome 21. Further, numerous
studies suggest that there are complex interactions
involving the genetic material on this chromosome. Such
interactions are believed to involve the production of
precursor proteins, proteases and protease inhibitors.

10   Further, it is believed that in individuals suffering
from Alzheimer's disease these interactions are somehow
skewed so that an unusually high content of β-amyloid
core protein is produced and/or deposited in the brain.
The high β-amyloid core protein concentration could be

15   the result of a variety of biochemical activities
including the overproduction of such proteins and/or the
inability to cleave sufficient numbers of such proteins
once cleaved.

            The transgenic mammals of the present invention
20   will provide insights with respect to how and where these
interactions occur and thus provide more useful models
for testing the efficacy of certain drugs in preventing
or reducing the accumulation of β-amyloid core protein in
the brain. The transgenic non-human mammals of the

25   present invention include recombinant genetic material
comprised of specific segments of β-amyloid precursor
proteins which segments are fused to specific promoters
capable of expressing the protein in specific tissues
such as nerve tissues generally and/or specific types of

30   nerve tissue, e.g., the brain.

35

-10-

3. Summary of the Invention

The invention provides a means for elucidating the molecular mechanisms involved in the synthesis of and, more importantly, inhibiting the synthesis and
5   deposition of β-amyloid protein (most importantly, in the brain) by inhibiting production and/or increasing cleavage after production. Cloned recombinant or synthetic DNA sequences related to the pathology of Alzheimer's disease are injected into fertilized
10 mammalian eggs (preferably mice eggs). The injected eggs are implanted in pseudopregnant females and are grown to term to provide transgenic mice whose cells express proteins related to the pathology of Alzheimer's disease. The injected sequences are constructs having promoter
15 sequences connected so as to express the desired protein in specific tissues of the transgenic mammals.

It is believed that β-amyloid core protein A99 and A42 (as defined herein) are first expressed in the form of precursor proteins A695 and/or A751. The
20 precursor proteins may be subjected to the action of a protease enzyme which allows for the formation of β-amyloid core proteins, which, when produced in sufficient amounts, allows for the formation of plaques associated with Alzheimer's disease. An important aspect
25 of the present invention relates to sequences which code for the production of any of A42, A99, A695, and A751 in specific cells (e.g., nerve cells) and to a sequence which codes for a protease inhibitor, which inhibits the action of the proteolytic enzyme which may be responsible
30 for converting precursor β-amyloid proteins into β-amyloid core proteins or which may inhibit a proteolytic enzyme (or enzymes) directed to catabolize or degrade the β-amyloid core protein.

In connection with the present invention
35 nucleotide sequences which encode for the production of

specific β-amyloid precursor proteins and β-amyloid core proteins are linked to specific promoter sequences. The promoter sequences are carefully chosen so that some sequences express the nucleotide sequence they are

5   attached to in all types of tissues whereas other promoter sequences only express the nucleotide they are attached to when the promoter is present within specific types of cells of a specific type of tissue e.g., nerve tissue. By producing such transgenic mice, additional

10   information can be obtained with respect to how and where β-amyloid core proteins are produced (and sometimes degraded) and deposited in an Alzheimer's diseased brain. Using this additional information regarding the mechanism and location of the production (and possible degradation)

15   and deposition of β-amyloid core proteins, therapeutic agents can be more effectively tested with respect to their ability to prevent the production and/or deposition of such proteins and thus alleviate or prevent Alzheimer's disease.

20   Further, an important aspect of this invention is the production of transgenic mammals which include DNA sequences that encode for analogs of a 57-amino acid protease inhibitor, which analogs contain at least one amino acid substitution which is effective to yield an

25   inhibitor having altered protease specificity. The transgenic mammals of this invention provide useful models for studying the _in vivo_ relationships of the proteins to each other and to other compounds being tested for their usefulness in treating Alzheimer's

30   disease.

     3.1.   Objects, Features and Advantages
            A primary object of this invention is to
     provide a transgenic non-human mammal whose cells include

35   a recombinant DNA sequence coding for cell type specific

expression of β-amyloid proteins or analog thereof, which
mammals can be used for the study of the etiology of
Alzheimer's disease and the efficacy of drugs in treating
the disease.

5   An advantage of the present invention is that
it provides an _in vivo_ means for studying the effects of
expressing different proteins in different tissues (e.g.,
nerve tissue including specific types of nerve tissues
and non-nerve tissue) vis-a-vis the synthesis of

10   β-amyloid protein and the formation of plaques associated
with Alzheimer's disease.

          Another object of the invention is to provide a
transgenic non-human mammal whose cells include a
recombinant DNA sequence comprising a cell specific

15   promoter sequence and a sequence coding for one or more
β-amyloid precursor proteins alone or with certain
protease inhibitor proteins which can be used for the
study of the etiology of Alzheimer's disease.

          Yet another object of the invention is to

20   provide transgenic mice which have in their cells unique
promoter/coding sequences which ubiquitously express
β-amyloid precursor protein in nerve tissue and nerve
tissue subtypes and/or all types of tissue.

          A further aspect of the invention relates to

25   synthesis and use of promoter/coding constructs which
express the β-amyloid precursor proteins alone or in
combination with inhibitor proteins in various tissues of
transgenic mammals incorporating such constructs in their
genome.   A feature of the transgenic mammals of the

30   invention is that they provide both prognostic and
diagnostic means for the study of Alzheimer's disease and
for determining the efficacy of pharmaceutical drugs in
treating Alzheimer's disease in a test subject.
          Initially, the transgenic mice are used as standards to

35   identify one or more candidate compounds capable of

metabolizing the β-amyloid protein (or preventing its formation) which is associated with a predisposition to Alzheimer's disease.

Yet another aspect of the invention relates to
5   a transgenic mammal incorporated with recombinant DNA sequences comprised of tissue specific promoter sequences and sequences coding for the expression of a 57 amino acid protease inhibitor and/or analogs thereof including a promoter specifically capable of expressing the
10   inhibitor in nerve tissue.

Other objects, advantages and features of the present invention include providing transgenic non-human mammals which provide information regarding the mechanism and location of β-amyloid core protein production and
15   disposition as well as providing in vivo models for testing drugs capable of interfering with or preventing such production and/or disposition.

These and other objects, features and advantages of the invention will become more fully
20   apparent when the following detailed description of the invention is read in conjunction with the accompanying figures.

4.   Brief Description of the Drawings
25   Figure 1, which includes 1-1, 1-2, 1-3 and 1-4, shows the base sequence of a cDNA clone, designated λAPCP168i4, which encodes amino acids 1-751 of β-amyloid precursor protein. The 168 bp insert, which distinguishes this clone from the Kang et al. sequence,
30   is underlined.

Figure 2 shows the amino acid sequence of A99.

Figure 3 shows the amino acid sequence of A42 corresponding to the β-amyloid core protein.

Figure 4 shows the construction strategy for
35   NSE promoter linked to A42 and to A99.

Figure 5 shows the construction strategy for
NSE promotor linked to A695 and to A751.

Figure 6 shows the construction strategy for MT
promotor linked to A42 and to A99.

5   Figure 7 shows the construction scheme for a
mammalian cell expression vector for the expression of
MT-A751 and MT-A695.

Figure 8 includes 8-1 and 8-2 and shows the
relatedness of the peptide encoded by the λAPCP16814 168

10   bp insert to a superfamily of proteins many of whose
members exhibit inhibitory activity for serine proteases.

Figure 9 shows the NSE promoter sequence and a
PCR Amplification Scheme for NSE.

Figure 10 shows a construction scheme for a

15   mammalian cell expression vector for β-amyloid
expression/selection of β-amyloid core constructs.

Figure 11A shows a schematic diagram of the
transgene NSE-A751.

Figure 11B shows a Southern blots of DNA taken

20   from wild-type and transgenic mice.

Figure 12 (A, B, C and D) show immunoperoxidase
staining of human and mouse brain.

Figure 13 (A, B, C, D and E) are
photomicrographs of immunoreactive deposits in NSE-A751

25   brains.

5.   **Detailed Description of Preferred Embodiments**

Before the present transgenic mice and process
for making and using such to test drugs are described, it

30   is to be understood that this invention is not limited to
the particular processes and materials described as such
methods and materials may, of course, vary. It is also
to be understood that the terminology used herein is for
the purpose of describing particular embodiments only,

35   and is not intended to be limiting since the scope of the

present invention will be limited only by the appended
claims.

It must be noted that as used in this
specification and the appended claims, the singular forms
5   "a", "an" and "the" include plural referents unless the
context clearly dictates otherwise. Thus, for example,
reference to "a clone" or "a sequence" includes mixtures
of clones or sequences of the type described, reference
to "an amyloid protein" includes reference to mixtures of
10   such proteins of the type described, and reference to
"the transgenic mouse" includes different species of such
mice and so forth.

     5.1. Definitions

15   The terms "core protein" and β-amyloid core
protein" mean a 99 amino acid protein as is shown in
Figure 2 and fragments thereof such as the 42 amino acid
sequence as shown in Figure 3. Such proteins are also
referred to herein as A99 and A42. A 42 amino acid core
20   protein is described by Masters, C.L., et al. Proc Natl
Acad Sci USA (1985) 82:4245-4249, herein referred to as
"Masters, et al."

     "A99" is a symbol representing the C-terminal
99 amino acids of the β-amyloid precursor and is
25   considered a "core protein".

     "A42" refers to a core protein of the β-amyloid
precursor. The N-terminus is the N-terminus of the 42
amino acid core and therefore contains the entire core
domain. As used throughout this application, the term
30   A42 corresponds to the 42 amino acid core sequence of the
β-amyloid precursor protein.

     "β-amyloid related protein" is defined herein
as: (1) a 751 amino acid protein as shown in Figure 1;
(2) a 695 amino acid protein as shown in Figure 1 minus
35   the 57 underlined amino acids; (3) the 57 amino acid

sequence underlined in Figure 1; and (4) analogs of any
of (1)-(3) and fragments thereof including, but not
limited to, A99 and A42. As an example, this term is
used to refer to the protein described by Kang, J. et
5   al., Nature (1987) 325:733-736, herein referred to as
"Kang, et al." which contains the β-amyloid core protein
within its structure at amino acid 597 of a 695 amino
acid protein.

        "β-amyloid precursor protein" includes a
10   subgroup of the "β-amyloid related proteins" defined
above. Such precursor proteins were first disclosed by
"Kang et al." and are produced naturally as larger
precursors proteins of the "β-amyloid core protein"; the
751 amino acid sequence is the most notable example of a
15   precursor protein as used in connection with the
invention.

        "Immunogenic β-amyloid core peptide" or
"immunogenic β-amyloid-related peptide" refer to peptides
whose amino acid sequences match those of some region of
20   the β-amyloid core protein or β-amyloid precursor
protein, and which are capable of provoking an antibody
response in an immunized animal. Examples of such
proteins are described in detail by Kitaguchi et. al.,
Biochemical and Biophysical Research Communications, Vol.
25   166, No. 3, Feb. 14, 1990.

        "Genetic predisposition to Alzheimer's disease"
refers to an identifiable genetic mutation or alteration
found in the genomes of individual's with Alzheimer's
disease, or those individuals destined to develop
30   Alzheimer's disease, but not normal (nondiseased)
individuals.

        "A4i" as used herein refers to the 57 amino
acid sequence underlined in Figure 1. The "A4i" may be a
polypeptide corresponding to the novel serine protease
35   inhibitor encoded by the polynucleotide derived from the

bacteriophage λAPCP168i4. The A4i polypeptide is not necessarily physically derived from the expression product of this bacteriophage, but may be generated in any manner, including peptide synthesis, recombinant DNA

5   techniques or a combination thereof. "Corresponding" means homologous to or substantially equivalent to the designated sequence.

"Genetic material" is a material containing any DNA sequence or sequences either purified or in a native

10   state such as a fragment of a chromosome or a whole chromosome, either naturally occurring or synthetically or partially synthetically prepared DNA sequences, DNA sequences which constitute a gene or genes and gene chimeras, e.g., created by ligation of different DNA

15   sequences. Genetic material does not include DNA sequences incorporated in or carried by a plasmid, virus or phage.

"Exogenous genetic material" is a genetic material not obtained from or does not naturally form a

20   part of the specific germ cells or gametes which form the particular zygote which is being genetically transformed.

"DNA sequence" is a linear sequence comprised of any combination of the four DNA monomers, i.e., nucleotides of adenine, guanine, cytosine and thymine,

25   which codes for genetic information, such as a code for an amino acid, a promoter, a control or a gene product. A specific DNA sequence is one which has a known specific function, e.g., codes for a particular polypeptide, a particular genetic trait or affects the expression of a

30   particular phenotype.

"Gene" is the smallest, independently functional unit of genetic material which codes for a protein product or controls or affects transcription and comprises at least one DNA sequence.

35

"Genotype" is the genetic constitution of an organism.

"Phenotype" is a collection of morphological, physiological and biochemical traits possessed by a cell 5 or organism that results from the interaction of the genotype and the environment.

"Phenotypic expression" is the expression of the code of a DNA sequence or sequences which results in the production of a product, e.g., a polypeptide or 10 protein, or alters the expression of the zygote's or the organisms natural phenotype.

"Zygote" is a diploid cell having the potential for development into a complete organism. The zygote can result from parthenogenesis, nuclear transplantation, the 15 merger of two gametes by artificial or natural fertilization or any other method which creates a diploid cell having the potential for development into a complete organism. The origin of the zygote can be from either the plant or animal kingdom.

20   "Parthenogenesis" is any technique that allows for the development of a female or male gamete into a cell and its development into an organism, which technique is different from the natural development of female and male gametes.

25

       5.2.   Disclosure Overview and Organization
              In order to disclose and describe this invention in an organized fashion, particular aspects of the invention are described in different sections. The 30 definition of terms has been given above in Section 5.1.

              The following Section 5.3 describes the various DNA sequences which express different proteins, precursor proteins, and inhibitors, as well as promoter sequences 35 which are fused to these sequences in order to obtain

expression of the protein in particular tissues. These DNA sequences are described in part by biological deposits made in connection with this disclosure and by reference to the attached figures.

5   Section 5.4 of the disclosure describes protocols by which it was possible to confirm that the sequence described in Section 5.3 could be expressed to produce proteins.

Section 5.5 describes eight different
10 promoter/A4 sequence constructs and, with reference to Figure 4 through Figure 7, describes the synthesis of the constructs.

Section 5.6 describes methods of producing the transgenic mice of the invention utilizing the DNA
15 sequences, constructs and other information described in the above sections.

Various methods and materials used in connection with the different aspects of the invention are described in Section 5.7.

20   Detailed Examples, the Claims and Abstract follow.

    5.3. DNA Sequences

In order to describe the transgenic mice of the
25 present invention, it is necessary to describe certain DNA sequences. For example, it is important to describe the DNA sequence which encodes a β-amyloid precursor protein comprising the nucleotide sequence and corresponding, deduced amino acid sequence set forth in
30 Figure 1. This DNA sequence encodes an approximately 82,610 dalton protein containing β-amyloid-related core protein.

As a first step toward producing the transgenic mice of the present invention, the β-amyloid protein cDNA
35 sequence, set forth in Figure 1, can be obtained in any

manner known to those skilled in the art. One method of
obtaining A751 is by isolating it from bacteriophage
containing the cDNA clone λAPCP168i4. This human
fibroblast cDNA clone known as λAPCP168i4 was deposited

5   at ATCC on July 1, 1987 and has accession No. 40347.
        The 168 basepair insert (underlined in
Figure 1) interrupts the codon for Val_{289} of the Kang
et al. sequence, resulting in the loss of this amino acid
from the λAPCP168i4 protein. The 168 basepair insert,

10   together with the 3 basepairs gained from the interrupted
Val_{289} codon, encode 57 new amino acids, which are
underlined in Figure 1. Downstream of this insertion, at
codon 653 of Figure 1, lies the amino-terminal aspartate
of the β-amyloid core protein described by Masters et al.

15   A unique feature of the transgenic mice of the
present invention relates to including cell specific
promoters in front of sequences which encode A42, A99,
A695, A751 and A4i (note that A4i is the 57 amino acid
insert found in λAPCP168i4).

20   The ability of the transgenic mice to
selectively express these particular peptides (in
specific types of cells), including any fragments
thereof, distinguishes the present transgenic mice from
others.

25   The cloned recombinant and/or synthetic DNA
sequences used in connection with the present invention
are sequences which are encoded for the production of a
biologically active, refolded protein, which protein is
preferably selected form the group consisting of:

30

A4 Name	Description	Sequence in Fig.
A42	β-amyloid core domain	3
A99	β-amyloid carboxy tail	2
A695	β-amyloid precursor protein	1
A751	precursor plus inhibitor	1
A4i	protease inhibitor	1

The A4i is underlined in Figure 1 and mice which
incorporate a sequence which expresses A4i generally or
in specific cells are part of the present invention
(i.e., notwithstanding cell specific promoters) as the
5   A4i protein may itself be a valuable compound in
preventing or reducing the effects of Alzheimer's
disease. Transgenic animals containing sequences
expressing A4i are not heretofore known.

10   5.4. Protein Production
         Preferred cDNA clones used in making the
transgenic mice include coding sequences which may be
expressed to obtain any one of A42, A99, A695, A751 and
A4i. These sequences are first inserted in a suitable
15 expression vector for replication and to confirm
production of protein.
         Briefly, an E. coli expression vector,
designated pAPCP118-3, was constructed for the expression
of a fusion protein consisting of amino acid residues 655
20 to 751 set forth in Figure 1. The construction of
pAPCP118-3 was accomplished by joining the following
three fragments: (1) a plasmid backbone (consisting of
pBR322 replication functions, an ampicillin resistance
gene, the tryptophan promoter and operator, a ribosome
25 binding site, DNA encoding the seven amino terminal
codons of the β-galactosidase structural gene followed by
six threonine residues, and transcription termination
signals); (2) an EcoRI-HaeII fragment encoding amino acid
residues 655-728 of the Figure 1 sequence; and (3) a
30 synthetic fragment encoding amino acid residues 729-751
of the Figure 1 sequence, followed by a stop codon.
         The resulting vector was used to transform E.
coli W3110 and expression of the fusion protein was
induced by reducing the tryptophan concentration followed
35 by the addition of 3-β-indoleacrylic acid. The resulting

protein can be purified using conventional purification
techniques and the resulting purified material is
available for use in the production of antibodies for
diagnostic assays.

5   The complete coding sequence of the β-amyloid
precursor protein set forth in Figure 1 was subcloned in
two fragments from the deposited λAPCP168i4 clone and
prepared for insertion into pSC11 or pUV1 vaccinia virus
expression vectors. Briefly, an approximately 1.06
10   kilobase (kb) EcoRI fragment, spanning amino acid
residues 655-751 of the protein illustrated in Figure 1,
was cloned into EcoRI-digested plasmid pGEM-3^{TM}
(available from Promega Biotec) to create an intermediate
vector designated p4BI. Subsequently p4BI was digested
15   with HindIII to remove much of the 3' noncoding sequence
of the β-amyloid-related sequence. The resulting vector
p4BWRI was digested with EcoRI and treated with calf
intestinal alkaline phosphatase prior to ligation to the
2088 bp EcoRI fragment derived from λAPCP168i4 to form
20   p4T4B. This plasmid was digested with SmaI and XmnI to
generate a 2678 bp fragment spanning the complete protein
encoding sequence set forth in Figure 1.

          The gene encoded by this SmaI-XmnI fragment was
inserted into one of the two well-known vaccinia viral
25   vectors, pSC11 and pUV1, for subsequent expression of the
β-amyloid precursor protein in CV-1 monkey kidney cells
using a eucaryotic transient expression system as
described by Cochran, M.A., et al., Proc Natl Acad Sci
USA (1985) 82:19-23. More commonly, these vectors are
·30 used for in vivo protein and antibody production in
animals after its sequences have· been inserted into the
vaccinia virus genome.

          Similarly, mammalian vectors can be utilized for
expression of the β-amyloid core protein or β-amyloid
35   precursor proteins described herein. A useful mammalian

vector utilizing human β-actin as a promoter is shown
within Figure 10. The details with respect to a
description of the vector shown within Figure 10 are as
follows:

5   Base pairs 1-4300 are the 4.3 kb EcoRI-AluI
fragment from the human β-actin gene isolate pl4Tβ-17
(Leavitt et al., Mol. Cell. Biol. (1984) 4:1961-1969)
           For sequencing details of the promoter, see Ng
et al., Mol. Cell. Biol. (1985) 5:2720-2732. The cap
10   site, 5' untranslated region and IVS 1 positions are
indicated. There is no ATG codon present in the 5'UT nor
in the poly-linker region from the 3' splice site to the
BamHI site.
           Base pairs 4300-4320 are in part derived from
15   pSP64 poly-linker (Melton et al., Nucl. Acids Res. (1984)
12:7035-7056).
           Base pairs 4320-6600 are derived from pcDV1
(Okavama & Berg, Mol. Cell. Biol. (1983) 3:280-289) and
contains the pBR322 Amp^R gene and bacterial origin plus
20   the SV40 late region polyadenylation signal.
           Base pairs 6600-10000 are the PvuII-EcoRI
fragment from pSV2-neo (Southern & Berg, J. Mol. App.
Genet. (1982) 1:327-341) containing the bacterial neo
gene linked to the SV40 ori plus early promoter.
25   Direction of transcription is as indicated. The vector
can be used for efficient protein expression in CHO
cells.
           An example of another potentially useful vector
is plasmid phGH-SV (10) (a plasmid described in EPA
30   217,822, published 15 April 1987, and incorporated herein
by reference) contains a pUC8 plasmid backbone, hMT-IIa
gene promoter and regulator elements, SV-40 DNA promoter
and enhancer elements, and the coding portions of the hGH
gene and 3' regulatory sequences. This plasmid can be
35   digested with BamHI and SmaI and treated with BamHI

linkers to delete the human growth hormone protein
encoding sequence and leaving the 3'-noncoding sequences
and regulatory elements attached to the plasmid backbone.
This approximately 5100 base pair DNA piece is gel

5   purified and ligated to BamHI linkers.

Digestion with BamHI, repurification of the DNA
fragment and subsequent ligation result in a plasmid
designated pMTSV40 polyA Bam which contains the
structural and regulatory elements comprising a mammalian

10   cell expression vector. After BamHI digestion of pMTSV40
polyA BamHI and repair in the presence of DNA polymerase
I and all four dNTPs, this vector is available for
insertion of the ~2678 bp SmaI-XmnI restriction fragment
of plasmid p4T4B. The vector can then be used for

15   efficient protein expression in CHO cells.

5.5.   <u>Promoter/A4 Sequence Fusion Constructs</u>

Eight different fusion constructs were prepared
by fusing one of two different promoters to different DNA

20   sequences which code for β-amyloid precursor proteins.
The promoter sequences used were mouse metallothionein-I
(MT) and rat neural-specific enolase (NSE). These
promoters or other useful promoters known to those
skilled in the art can be linked to various A4 sequences

25   of the type described above.

Some useful neural-specific promoters which can
be used to create constructs and inserted into transgenic
mice in connection with the present invention are as
follows:

30   (1) <u>Neurofilament M or L promoters</u>. These
promoters demonstrate a high level of expression and are
found in connection with the most abundant neural
protein. They are characterized by CNS/PNS neuronal-
specific expression and have been used in connection with

35   transgenic expression. The mouse gene for this promoter

is a published sequence and the isolation of the promoter
region is necessary in order to use the promoter in
connection with the present invention.

       (2)   <u>Glial fibrillary acidic protein (GFAP)</u>

5   promoter sequences. Such promoters are characterized by
murine specificity and CNS/PNS glial-specific expression.
The promoter has been characterized and is available.

       (3)   <u>Growth associated protein 43 (GAP 43)</u> is
also characterized by CNS/PNS neuronal-specific

10   expression. The promoter is expressed developmentally
and upon induced injury. The promoter within a rat has
been characterized and is available.

       (4)   <u>Nerve growth factor (NGF)</u> promoters are
characterized by PNS developmental expression and CNS

15   maintained expression in the hippocampus and cortex which
are the same areas afflicted by Alzheimer's disease. The
mouse gene promoter is published and isolation of this
promoter is possible and necessary for use in connection
with the present invention.

20   (5)   <u>The JC Virus T antigen</u> can be used. The
human papilloma virus has neuronal tropism. The T Ag
promoter is characterized and is available.

       (6)   <u>pp60^{c-src}</u> demonstrates 10x higher
expression levels in CNS as compared with its expression

25   in non-neuronal cells. The regions of the CNS expression
are confined to specific brain regions and the promoter
has been characterized.

       (7)   <u>N-CAM - Neural cell adhesion molecule</u>
demonstrates murine neuronal-specific expression.

30   While not wishing to be bound to any particular
theory regarding the pathology of Alzheimer's disease, it
is postulated that the ratio of the amount of A751 to the
amount of A659 precursors which are unique to the nervous
system may be in a state of imbalance, giving rise to

35   Alzheimer's pathology. This imbalance (resulting from

over- or under-expression of one precursor) might create
a condition for amyloid to form in abundance and create
the plaques. Based on this theory, constructs were
designed to express either the A751 or A695 β-amyloid
5   protein precursors.

Alternatively, Alzheimer's pathology may result
from inadequate or incomplete catabolism of the carboxy
tail of the β-amyloid precursor protein (A99 sequences)
which is released when A695 and A751 are processed to
10   soluble extracellular proteins (see Weidemann, A., et
al., Cell. (1989) 57:115-126). Based on this theory,
constructs were designed to express the carboxy tail
(A99) or the β-amyloid core protein (A-42). All
constructs were designed to permit expression of the
15   construct either specific to neural tissues (NSE) or
ubiquitously expressed in all types of tissue (MT
promoters).

The following fusion constructs are preferred
examples of constructs useful in connection with the
20   present invention:

Promoter	A4 Sequence	Figure
NSE	A42 core domain	4
NSE	A99 carboxy tail	4
NSE	A751	5
NSE	A695	5
NSE	A4i	
MT	A751	6
MT	A695	6
MT	A42 core domain	7
MT	A99 carboxy tail	7
MT	A4i	

30

As indicated above, Figures 4-7 and 10 detail
the cloning strategies used for producing the different
fusion constructs. It should be pointed out that β-actin
A42 and β-actin A99 plasmids served as the source for the
35   A42 and A99 sequences, respectively, for the

constructions. Complete coding fidelity was maintained
in the A42 and A99 constructs; each was simply proceeded
by a methionine initiator codon.

The β-actin A751 and β-actin A695 vectors were
5   used to prepared the other NSE promoter constructs. All
of the plasmid constructions have been confirmed by
restriction map analysis. All of the constructs have
been definitively confirmed by DNA sequence analysis of
the cloning junctions.

10   The MT-I promoter used for the fusion constructs
was derived using synthetic oligonucleotides. The MT-1
promoter is sequenced and described in detail by Swanson,
L.W. et al., Novel Developmental Specificity in the
Nervous Systems of Transgenic Animals Expressing Growth
15   Hormone Fusion Genes, Nature (1985) 317:363-366,
incorporated herein by reference to describe the MT-I
promoter. The MT-I promoter region encompasses 373
basepairs and can be constructed by synthesis and
ligation of DNA oligomers.
20   Five pairs of oligomers 70-80 nucleotides in length are
used. The resultant 373-basepair fragment can be cloned
and sequenced. The MT-I promoter can also be isolated by
selectively amplifying this region of the mouse genome
using the polymerase chain reaction (PCR) method. The
25   amplified fragment can be cloned and characterized by DNA
sequence analysis.

The MT-I promoter is sequenced and described in
detail by Swanson, L.W. et al., Novel Developmental
Specificity in the Nervous System of Transgenic Animals
30   Expressing Growth Hormone Fusion Genes, Nature (1985)
317:363-366, incorporated herein by reference to disclose
the MT-I promoter.

Two different approaches can also be used to
isolate the rat neuronal-specific enolase promoter (NSE).
35   The nucleotide sequence of the promoter region has been

published. Accordingly, it is possible to design
synthetic oligonucleotides for use in PCR and for
screening genomic libraries. Oligonucleotide primers
were used to selectively amplify the region of interest

5   using PCR and rat genomic DNA. A 1.15 kilobasepair
fragment was isolated and cloned. The same
oligonucleotide has been employed as a probe to screen
rat genomic libraries.

The MT-I promoter was proven functional by

10   assessing the transient expression of a construct bearing
the CAT reporter gene (that is, the reporter gene
chloramphenicol acetyltransferase), after transfection of
the DNA into CHO cells.

The NSE promoter can also be isolated from a

15   genomic clone purified from a buffalo rat DNA library
using oligonucleotide probes designed from the published
sequence of the promoter (Sakimura et al., Gene (1987)
60:103-113) which is incorporated herein by reference to
disclose such a promoter.

20   A promoter region fragment of 2.3 kilobases
including a 1.2 kilobase intron in the 5'-untranslated
region was prepared from 11 kb clone using polymerase
gene reaction amplification. The PCR strategy and
oligonucleotide primers used are described in Figure 9.

25   The 3'-terminal primer for the NSE promoter fragment
plus intron-harbored 2 nucleotide substitutions to create
a NcoI site for cloning purposes, as well as to introduce
the initiator methionine codon for the A4 sequences. It
is pointed out that polymerase chain reaction

30   amplification is reported to have a low error rate.

     5.6. Transgenic Organisms
             The transgenic organisms of the invention all
include within a plurality of their cells a cloned

35   recombinant or synthetic DNA sequence which is believed

to relate to the pathogenesis of Alzheimer's Disease.
More specifically, the transgenic organisms contain
specific sequences of exogenous genetic material, such as
the sequences described above in detail which are

5   comprised of a tissue specific promoter sequence and a
sequence which encodes for production of a β-amyloid
precursor protein. Since it is possible to produce
transgenic organisms of the invention utilizing one or
more of the above-described sequences, a general

10   description will be given of the production of transgenic
organisms by referring generally to exogenous genetic
material. This general description can be adapted by
those skilled in the art in order to incorporate the
above-described specific DNA sequences into organisms and

15   obtain expression of those sequences utilizing the
methods and materials described below. For more details
regarding the production of transgenic organisms, and
specifically transgenic mice, refer to U.S. Patent
4,873,191, issued October 10, 1989 (incorporated herein

20   by reference to disclose methods producing transgenic
mice), and to the numerous scientific publications
referred to and cited therein.

     The exogenous genetic material may be placed in
either the male or female pronucleus of the zygote. More

25   preferably, it is placed in the male pronucleus as soon
as possible after the sperm enters the egg. In other
words, right after the formation of the male pronucleus
when the pronuclei are clearly defined and are well
separated, each being located near the zygote membrane.

30   The male pronucleus of a fertilized mouse egg is the
preferred site for addition of the exogenous genetic
material of the present invention.

     It is most preferred that the exogenous genetic
material be added to the male DNA complement of the

35   zygote prior to its being processed by the ovum nucleus

-30-

or the zygote female pronucleus. It is thought that the ovum nucleus or female pronucleus release molecules which affect the male DNA complement, perhaps by replacing the protamines of the male DNA with histones, thereby

5 facilitating the combination of the female and male DNA complements to form the diploid zygote.

Thus, it is preferred that the exogenous genetic material be added to the male complement of DNA or any other complement of DNA prior to its being affected by

10 the female pronucleus. For example, the exogenous genetic material is added to the early male pronucleus, as soon as possible after the formation of.the male pronucleus, which is when the male and female pronuclei are well separated and both are located close to the cell

15 membrane. Alternatively, the exogenous genetic material could be added to the nucleus of the sperm after it has been induced to undergo decondensation. Sperm containing the exogenous genetic material could then be added to the ovum or the decondensed sperm could be added to the ovum

20 with the exogenous genetic material being added as soon as possible thereafter.

For the purposes of this invention a zygote is essentially the formation of a diploid cell which is capable of developing into a complete organism.

25 Generally, the zygote will be comprised of an egg containing a nucleus formed, either naturally or artificially, by the fusion of two haploid nuclei from a gamete or gametes. Thus, the gamete nuclei must be ones which are naturally compatible, i.e., ones which result

30 in a viable zygote capable of undergoing differentiation and developing into a functioning organism. Generally, a euploid zygote is preferred. If an aneuploid zygote is obtained, then the number of chromosomes should not vary by more than one with respect to the euploid number of

35 the organism from which either gamete originated.

In addition to similar biological
considerations, physical ones also govern the amount of
exogenous genetic material which can be added to the
nucleus of the zygote or to the genetic material which

5   forms a part of the zygote nucleus. If no genetic
material is removed, then the amount of exogenous genetic
material which can be added is limited by the amount
which will be absorbed without being physically
disruptive. Generally, the volume of exogenous genetic

10   material inserted will not exceed about 10 picoliters.
The physical effects of addition must not be so great as
to physically destroy the viability of the zygote. The
biological limit of the number and variety of DNA
sequences will vary depending upon the particular zygote

15   and functions of the exogenous genetic material and will
be readily apparent to one skilled in the art, because
the genetic material, including the exogenous genetic
material, of the resulting zygote must be biologically
capable of initiating and maintaining the differentiation

20   and development of the zygote into a functional organism.
The number of copies of the DNA sequences which
are added to the zygote is dependent upon the total
amount of exogenous genetic material added and will be
the amount which enables the genetic transformation to

25   occur. Theoretically only one copy is required; however,
generally, numerous copies are utilized, for example,
1,000-20,000 copies of a gene, in order to insure that
one copy is functional. As regards the present
invention, there is generally an advantage to having more

30   than one functioning copy of each of the inserted
exogenous DNA sequences to enhance the phenotypic
expression of the exogenous DNA sequences (i.e., to
obtain ubiquitous expression of the β-amyloid related
precursor proteins and protease inhibitor proteins).

35

Any technique which allows for the addition of the exogenous genetic material into nucleic genetic material can be utilized so long as it is not destructive to the cell, nuclear membrane or other existing cellular
5   or genetic structures. The exogenous genetic material is preferentially inserted into the nucleic genetic material by microinjection. Microinjection of cells and cellular structures is known and is used in the art.

The transgenic mammals produced in accordance
10   with the present invention will include exogenous genetic material. The exogenous genetic material will be a DNA sequence which results in the production of a β-amyloid related protein. Further, the sequence will be attached to a promoter which promoter preferably allows the
15   expression of the β-amyloid related protein in a specific type of cell such as a nerve cell and may include a promoter which allows expression within a particular type of nerve cell.

In some preferred embodiments of the invention
20   the transgenic animal includes a cell specific promoter in connection with a DNA sequence which results in the production of a β-amyloid precursor protein. A number of examples of such precursor proteins are disclosed and described. As a particularly preferred example of a
25   transgenic mammal of the invention there is provided transgenic mammals which include DNA sequences capable of producing all or any of the proteins as described above in Section 5.5. The sequences are preferably included in connection with a promoter as also shown within Section
30   5.5. However, other promoters which allow for cell-specific expression and particularly nerve cell-specific and still more particularly certain types of nerve cell-specific expression are preferred embodiments of the invention. In yet another preferred embodiment of the
35   invention the transgenic mammal includes a sequence which

is capable of producing the A4i protein. This unique
sequence may be produced in connection with all types of
promoters. Transgenic mammals containing sequences
capable of expressing A4i proteins were not known prior

5   to the present invention. Particularly preferred
embodiments of the invention include sequences capable of
producing such A4i proteins which sequences are connected
to cell-type specific promoters such as nerve cell-
specific promoters and still more particularly in

10   connection with particular types of nerve cell-specific
promoters.

5.7. Methods and Materials

Most of the techniques which are used to

15   transform cells, construct vectors, extract messenger
RNA, prepare cDNA, and the like are widely practiced in
the art, and most practitioners are familiar with the
standard resource materials as well as specific
conditions and procedures. However, for convenience, the

20   following paragraphs may serve as a guideline.

5.7.a. Hosts and Control Sequences

Both procaryotic and eucaryotic systems may be
used to express the β-amyloid core and β-amyloid-related

25   sequences; procaryotic hosts are, of course, the most
convenient for cloning procedures. Procaryotes most
frequently are represented by various strains of_
E. coli; however, other microbial strains may also be
used. E. coli strains may secrete the β-amyloid core and

30   β-amyloid precursor proteins to the periplasm when the
genes encoding these proteins are fused to appropriate
signal peptides, and certain E. coli strains, for
example, a lipoprotein mutant strain such as JE5505
(Kanamari, T. Gene (1988) 66:295-300), will excrete the

35   chimeric proteins directly to the culture medium.

          Plasmid vectors which contain replication sites,
selectable markers and control sequences derived from a
species compatible with the host are used; for example,
E. coli is typically transformed using derivatives of
5   pBR322, a plasmid derived from an E. coli species by
Bolivar, et al., Gene (1977) 2:95. pBR322 contains genes
for ampicillin and tetracycline resistance, and thus
provides multiple selectable markers which can be either
retained or destroyed in constructing the desired vector.
10   Commonly used procaryotic control sequences which are
defined herein to include promoters for transcription
initiation, optionally with an operator, along with
ribosome binding site sequences, include such commonly
used promoters as the β-lactamase (penicillinase) and
15   lactose (lac) promoter systems (Chang, et al., Nature
(1977) 198:1056) and the tryptophan (trp) promoter system
(Goeddel, et al. Nucleic Acids Res (1980) 8:4057) and the
lambda derived P_L promoter and N-gene ribosome binding
site (Shimatake, et al., Nature (1981) 292:128).
20   Other procaryotic control sequences include
signal sequences which direct secretion of a protein to
the periplasm. Commonly used bacterial signal peptides
include the ompA (Kikuchi, et al., Nucleic Acids Res
(1981) 9:5671-5678) and phoA (Beck and Bremer, Nucleic
25   Acids Res (1980) 8:3011-3024) signal peptides which can
be fused to the protease inhibitor sequence of the
invention.
          In addition to bacteria, eucaryotic microbes,
such as yeast, may also be used as hosts. Laboratory
30   strains of Saccharomyces cerevisiae, Baker's yeast, are
most used although a number of other strains or species
are commonly available. Vectors employing, for example,
the 2 m origin of replication of Broach, J. R., Meth Enz
(1983) 101:307, or other yeast compatible origins of
35   replication (see, for example, Stinchcomb, et al., Nature

(1979) 282:39, Tschumper, G., et al., Gene (1980) 10:157
and Clarke, L., et al., Meth Enz (1983) 101:300) may be
used. Control sequences for yeast vectors include
promoters for the synthesis of glycolytic enzymes (Hess,
et al., J Adv Enzyme Reg (1968) 7:149; Holland, et al.,
Biochemistry (1978) 17:4900). Additional promoters known
in the art include the promoter for 3-phosphoglycerate
kinase (Hitzeman, et al., J Biol Chem (1980) 255:2073).
Other promoters, which have the additional advantage of
transcription controlled by growth conditions and/or
genetic background are the promoter regions for alcohol
dehydrogenase 2, isocytochrome C, acid phosphatase,
degradative enzymes associated with nitrogen metabolism,
the alpha factor system and enzymes responsible for
maltose and galactose utilization. It is also believed
terminator sequences are desirable at the 3' end of the
coding sequences. Such terminators are found in the 3'
untranslated region following the coding sequences in
yeast-derived genes.

It is also, of course, possible to express genes
encoding polypeptides in eucaryotic host cell cultures
derived from multicellular organisms. See, for example,
Axel, et al., U.S. Patent No. 4,399,216. These systems
have the additional advantage of the ability to splice
out introns and thus can be used directly to express
genomic fragments. Useful host cell lines include VERO
and HeLa cells, and Chinese hamster ovary (CHO) cells.
Expression vectors for such cells ordinarily include
promoters and control sequences compatible with mammalian
cells such as, for example, the commonly used early and
late promoters from Simian Virus 40 (SV 40) (Fiers, et
al., Nature (1978) 273:113), or other viral promoters
such as those derived from polyoma, adenovirus 2, bovine
papilloma virus, or avian sarcoma viruses. The
controllable promoter, hMTII (Karin, M., et al., Nature

(1982) 299:797-802) may also be used. General aspects of
mammalian cell host system transformations have been
described by Axel (supra). It now appears, also that
"enhancer" regions are important in optimizing
5   expression; these are, generally, sequences found
upstream or downstream of the promoter region in
noncoding DNA regions. Origins of replication may be
obtained, if needed, from viral sources. However,
integration into the chromosome is a common mechanism for
10 DNA replication in eucaryotes.

          5.7.b. Transformations
          Depending on the host cell used, transformation
is done using standard techniques appropriate to such
15 cells. The calcium treatment employing calcium chloride,
as described by Cohen, S.N., Proc Natl Acad Sci (USA)
(1972) 69:2110, or the RbCl$_2$ method described in
Maniatis, et al., Molecular Cloning: A Laboratory
Manual (1982) Cold Spring Harbor Press, p. 254 and
20 Hanahan, D., J Mol Biol (1983) 166:557-580 may be used
for procaryotes or other cells which contain substantial
cell wall barriers. For mammalian cells without such
cell walls, the calcium phosphate precipitation method of
Graham and van der Eb, Virology (1978) 52:546, optionally
25 as modified by Wigler, M., et al., Cell (1979) 16:777-
785 may be used.
     Transformations into yeast may be carried out according
to the method of Beggs, J.D., Nature (1978) 275:104-109
or of Hinnen, A., et al., Proc Natl Acad Sci (USA) (1978)
30 75:1929.

35

5.7.c. Vector Construction

Construction of suitable vectors containing the
desired coding and control sequences employs standard
ligation and restriction techniques which are well

5 understood in the art. Isolated plasmids, DNA sequences,
or synthesized oligonucleotides are cleaved, tailored,
and religated in the form desired.

The DNA sequences which form the vectors are
available from a number of sources. Backbone vectors and

10 control systems are generally found on available "host"
vectors which are used for the bulk of the sequences in
construction. For the pertinent coding sequence, initial
construction may be, and usually is, a matter of
retrieving the appropriate sequences from cDNA or genomic

15 DNA libraries. However, once the sequence is disclosed
it is possible to synthesize the entire gene sequence in
vitro starting from the individual nucleotide
derivatives. The entire gene sequence for genes of
sizeable length, e.g., 500-1000 bp may be prepared by

20 synthesizing individual overlapping complementary
oligonucleotides and filling in single stranded
nonoverlapping portions using DNA polymerase in the
presence of the deoxyribonucleotide triphosphates. This
approach has been used successfully in the construction

25 of several genes of known sequence.

See, for example, Edge, M. D., Nature (1981) 292:756;
Nambair, K. P., et al., Science (1984) 223:1299; Jay,
Ernest, J Biol Chem (1984) 259:6311.

Synthetic oligonucleotides are prepared by

30 either the phosphotriester method as described by Edge,
et al., Nature (supra) and Duckworth, et al., Nucleic
Acids Res (1981) 9:1691 or the phosphoramidite method as
described by Beaucage, S.L., and Caruthers, M.H., Tet
Lett (1981) 22:1859 and Matteucci, M.D., and Caruthers,

35 M.H., J Am Chem Soc (1981) 103:3185 and can be prepared

using commercially available automated oligonucleotide
synthesizers. Kinasing of single strands prior to
annealing or for labeling is achieved using an excess,
e.g., approximately 10 units of polynucleotide kinase to
5   1 nmole substrate in the presence of 50 mM Tris, pH 7.6,
10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles
γ32P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Once the components of the desired vectors are
thus available, they can be excised and ligated using
10 standard restriction and ligation procedures.

Site-specific DNA cleavage is performed by
treating with the suitable restriction enzyme (or
enzymes) under conditions which are generally understood
in the art, and the particulars of which are specified by
15 the manufacturer of these commercially available
restriction enzymes. See, e.g., New England Biolabs,
Product Catalog. In general, about 1 mg of plasmid or
DNA sequence is cleaved by one unit of enzyme in about 20
ml of buffer solution; in the examples herein, typically,
20 an excess of restriction enzyme is used to insure
complete digestion of the DNA substrate. Incubation times
of about one hour to two hours at about 37°C are
workable, although variations can be tolerated. After
each incubation, protein is removed by extraction with
25 phenol/chloroform, and may be followed by ether
extraction, and the nucleic acid recovered from aqueous
fractions by precipitation with ethanol. If desired,
size separation of the cleaved fragments may be performed
by polyacrylamide gel or agarose gel electrophoresis
30 using standard techniques. A general description of size
separations is found in Methods in Enzymology (1980)
65:499-560.

Restriction cleaved fragments may be blunt ended
by treating with the large fragment of E. coli DNA
35 polymerase I (Klenow) in the presence of the four

deoxynucleotide triphosphates (dNTPs) using incubation
times of about 15 to 25 min at 20 to 25°C in 50 mM Tris
pH 7.6, 50 mM NaCl, 6 mM MgCl$_2$, 6 mM DTT and 0.1-1.0 mM
dNTPs. The Klenow fragment fills in at 5' single-
5   stranded overhangs but chews back protruding 3' single
strands, even though the four dNTPs are present.

If desired, selective repair can be performed by
supplying only one of the, or selected, dNTPs within the
limitations dictated by the nature of the overhang. After
10   treatment with Klenow, the mixture is extracted with
phenol/chloroform and ethanol precipitated. Treatment
under appropriate conditions with S1 nuclease or BAL-31
results in hydrolysis of any single-stranded portion.

Ligations are performed in 15-50 ml volumes
15   under the following standard conditions and temperatures:
for example, 20 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 10 mM
DTT, 33 μg/ml BSA, 10 mM-50 mM NaCl, and either 40 μM
ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for
"sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units
20   T4 DNA ligase at 14°C (for "blunt end" ligation).
Intermolecular "sticky end" ligations are usually
performed at 33-100 μg/ml total DNA concentrations (5-
100 nM total end concentration). Intermolecular blunt end
ligations are performed at 1 mM total ends concentration.
25   In vector construction employing "vector
fragments", the vector fragment is commonly treated with
bacterial alkaline phosphatase (BAP) or calf intestinal
alkaline phosphatase (CIP) in order to remove the 5'
phosphate and prevent self-ligation of the vector.
30   Digestions are conducted at pH 8 in approximately 10 mM
Tris-HCl, 1 mM EDTA using about 1 unit of BAP or CIP per
mg of vector at 60' for about one hour. In order to
recover the nucleic acid fragments, the preparation is
extracted with phenol/chloroform and ethanol
35   precipitated. Alternatively, religation can be prevented

in vectors which have been double digested by additional
restriction enzyme digestion and separation of the
unwanted fragments.

For portions of vectors derived from cDNA or

5   genomic DNA which require sequence modifications, site
specific primer directed mutagenesis may be used (Zoller,
M.J., and Smith, M. Nucleic Acids Res (1982) 10:6487-
6500 and Adelman, J.P., et al., DNA (1983) 2:183-193).
This is conducted using a primer synthetic

10   oligonucleotide complementary to a single stranded phage
DNA to be mutagenized except for limited mismatching,
representing the desired mutation. Briefly, the synthetic
oligonucleotide is used as a primer to direct synthesis
of a strand complementary to the phage, and the resulting

15   partially or fully double-stranded DNA is transformed
into a phage-supporting host bacterium. Cultures of the
transformed bacteria are plated in top agar, permitting
plaque formation from single cells which harbor the
phage.

20   Theoretically, 50% of the new plaques will
contain the phage having, as a single strand, the mutated
form; 50% will have the original sequence. The resulting
plaques are washed after hybridization with kinased
synthetic primer at a wash temperature which permits

25   binding of an exact match, but at which the mismatches
with the original strand are sufficient to prevent
binding. Plaques which hybridize with the probe are then
picked, cultured, and the DNA recovered.

30   5.7.d. Verification of Construction
In the constructions set forth below, correct
ligations for plasmid construction are confirmed by first
transforming E. coli strain MC1061 obtained from Dr. M.
Casadaban (Casadaban, M., et al., J Mol Biol (1980)

35   138:179-207) or other suitable host with the ligation

mixture. Successful transformants are selected by
ampicillin, tetracycline or other antibiotic resistance
or using other markers depending on the mode of plasmid
construction, as is understood in the art. Plasmids from

5   the transformants are then prepared according to the
method of Clewell, D.B., et al., Proc Natl Acad Sci
(USA) (1969) 62:1159, optionally following
chloramphenicol amplification (Clewell, D.B., J
Bacteriol (1972) 110:667). Several mini DNA preps are

10   commonly used, e.g., Holmes, D.S., et al., Anal Biochem
(1981) 114:193-197 and Birnboim, H.C., et al., Nucleic
Acids Res (1979) 7:1513-1523. The isolated DNA is
analyzed by restriction and/or sequenced by the dideoxy
nucleotide method of Sanger, F., et al., Proc Natl Acad

15   Sci (USA) (1977) 74:5463 as further described by Messing,
et al., Nucleic Acids Res (1981) 9:309, or by the method
of Maxam, et al., Methods in Enzymology (1980) 65:499.

                            6.0

                         EXAMPLES

20   The following examples are put forth so as to
provide those of ordinary skill in the art with a
complete disclosure and description of how to make the
DNA sequences, fusion constructs, proteins and transgenic

25   mammals of the invention and are not intended to limit
the scope of what the inventors regard as their
invention. Efforts have been made to ensure accuracy
with respect to numbers used (e.g., amounts, temperature,
etc.) but some experimental errors and deviation should

30   be accounted for. Unless indicated otherwise, parts are
parts by weight, temperature is in degrees C, and
pressure is at or near atmospheric.

35

-42-

6.1
EXAMPLE 1
Expression of β-Amyloid-Related Protein
(1-751) in Cultured Mammalian Cells

5   To facilitate the expression of β-amyloid
precursor protein in mammalian cells, a plasmid is
constructed such that the coding segment for the protein
is fused to a powerful regulated promoter derived from
the human metallothionine II (hMTII) gene. This procedure
10   is performed in two steps. First an expression vector
pMTSV40 polyA Bam was derived from phGH-SV(10) vector by
digestion of phGH-SV(10) with BamHI and SmaI restriction
enzymes, followed by incubation with DNA polymerase I
(Klenow fragment) in order to create blunt-ended
15   molecules. The blunt ends are subsequently ligated to
BamHI linkers, cut with BamHI, and religated to allow for
recircularization. This step removes all of the human
growth hormone genomic sequence from phGH-SV(10) except
for most of the 3' untranslated region of the mRNA and
20   genomic sequences encoding putative 3' transcriptional
stop and processing signals. For the mammalian cell
expression construct, pMTSV40 polyA Bam is BamHI-
digested, then incubated with all four nucleotide
triphosphates and with DNA polymerase I to create blunt
25   ends. This fragment is subsequently ligated with the
purified 2678 bp SmaI-XmnI fragment derived from p4T4B.
The recombinant molecules are introduced into MC1061 by
transformation.
        Chinese hamster ovary (CHO)-K1 cells are grown
30   in a medium composed of a 1:1 mixture of F12 medium and
DME medium with 10% fetal calf serum. The competent
cells are co-transformed with the recombinant expression
vector and pSU2:NEO (Southern, P., et al., _J Mol Appl_
Genet (1982) _1_:327-341). pSV2:NEO contains a functional
35   gene conferring resistance to the neomycin analog G418.

In the transformation, 500 ng of pSV2:NEO and 5 μg of the recombinant vector are applied to a 60 mm dish of CHO cells as a calcium phosphate-DNA co-precipitate as described by Graham, F.L. and Van der Eb, A.J. _Virology_

5   (1973) 52:456-467. Growth of the cells in the antibiotic G418 as described by Southern et al. will yield a pool of stably transfected CHO cells containing expression vector DNA with the capacity to express β-amyloid-related mRNA and protein.

10
                              6.2
                          EXAMPLE 2
        Expression of β-Amyloid Precursor in Mammalian Cells
                Outlined in Example 1 is the construction of an

15   expression system for the β-amyloid precursor protein (1-751) driven by the human promoter. A nearly identical construct was prepared using the purified 2548 bp SmaI-XmnI fragment derived from p4T4B from which 116 bp from the 5' untranslated region have been deleted. This

20   fragment was inserted into the SalI site behind the human promoter on a plasmid harboring the neomycin selectable marker for mammalian cell expression and the ampicillin resistance gene for selection of bacterial transformants. This vector, pHbAPr-1-neo, has been described by Gunning,

25   et al., (_Proc Nat'l Acad Sci USA_ (1987) 84:4831-4835) and has been modified to remove the EcoRI site from the body of the original vector and to substitute the original polylinker region with a new polylinker containing an EcoRI site in addition to the SalI, HindIII, and BamHI

30   cloning sites originally present. The modified vector is referred to as pAXneoR. The pAXneoR vector was linearized with SalI, the termini filled in using Klenow fragment of DNA polymerase to create blunt-ended molecules. The 2548 bp SmaI-XmnI β-amyloid fragment was

35   blunt-ligated into the vector using T4 ligase. The

recombinant molecules were introduced into E. coli MC1061
by transformation and a clone displaying the proper
orientation was amplified. A similar construction was
made using the 695 β-amyloid sequences described by Kang
et al. (supra) which places the 695 amyloid protein under
control of the human promoter.

600 μg total DNA of pAXneo/751 β-amyloid or
pAXneo/695 β-amyloid or an equal mass mixture of both
plasmid constructs were introduced into 10^7 CHO cells by
electroporation (Neumann, J Membrane Biol (1972) 10:279-
290; Zimmerman, Biophys J (1973) 13:1005-1013) using a
BTX Transfector 100, Bio-Rad sterile, disposal cuvettes
and a custom built cuvette holder. G418-resistant cells
receiving the exogenous DNA were selected by standard
protocols (Southern, 1982, supra) using 500 μg/ml G418
from Gibco.

The pool of positively transfected cells
resistant to G418 from each of the three transfections
was characterized with respect to β-amyloid precursor
protein expression. Approximately 2 x 10^6 cells from
each pool containing 5 ml of serum-free medium were
incubated at 37°C for 48 hr. The conditioned media was
removed and the protein precipitated by addition of
trichloroacetic acid to a final concentration of 10%.
Cells were harvested by scraping, washed in saline
buffered with phosphate and resuspended in 50 μl of
buffer for a 30-fold concentration. 25 μl of each sample
was loaded onto a 12.5% polyacrylamide gel (Laemmli,
Nature (1970) 277:680-685). The β-amyloid precursor was
detected by Western blot analysis (Towbin, Proc Nat'l
Acad Sci USA (1979) 76:4350-4354) using β-amyloid-
specific polyclonal antibodies generated by recombinant
vaccinia virus harboring the β-amyloid 751 cDNA by using
standard procedures. Typically, the majority of the
approximately 110,000 dalton β-amyloid precursor is found

to be released into the culture media and very small
amounts of the protein is cell-associated. This result
is in keeping with the hypothesis of Allsop, et al.,
(_Proc Natl Acad Sci USA_ (1988) _85_:2790-2794) proposing

5   that the β-amyloid protein is a secreted prohormone. The
apparent molecular weight of 110,000 daltons of the
recombinantly expressed β-amyloid protein is similar to
that observed by others (Dyrks, T., et al., _EMBO J_ (1988)
_7_(4):949-957) using _in vitro_ transcription/translation

10   systems and using cells in culture (Weidemann, A., et
al., _Cell_ _57_, 115-126 (1989).

6.3

EXAMPLE 3

15   Assay to Distinguish Genetic Variants of
β-Amyloid-Related Protein mRNA Species
    The ability to distinguish between genetic
variants of β-amyloid precursor protein mRNA species
using oligonucleotide probes is demonstrated herein. This

20   diagnostic assay can distinguish between two closely
related genetic variants of β-amyloid precursor proteins
or their mRNAs, and quantitate the relative levels of
expression of these proteins or mRNAs.
    Total cellular RNA or cytoplasmic RNA was

25   prepared from human cells in culture or human brain
tissue (Alzheimer's brain or normal brain) with or
without removal of nuclei (cytoplasmic or total,
respectively) by the guanidine thiocyanate/CsCl method as
described by Maniatis et al. RNA was fractionated by

30   oligo-dT cellulose chromatography, electrophoresed on a
formaldehyde agarose gel, and blot-transferred to
nitrocellulose (all as described in Maniatis et al.)
Filters were baked, prehybridized and hybridized to the
indicated probes according to standard protocols.

35

Oligonucleotide probes were end-labeled with
[^{32}P]-dCTP by incubation with terminal transferase
according to manufacturer's suggestions and as described
by Ponte et al. Nature 331,525,527 (11 February 1988)
which is incorporated herein by reference to disclose
such end-labeling. Actin insert was radiolabeled with
[^{32}P]-CTP by nick-translation. After hybridization, the
filters hybridized to oligonucleotides were washed at 1 x
SSC, 55°C. The filter hybridized to actin was washed at
0.1 x SSC at 55°C. Filters were then exposed to X-ray
film to produce the autoradiogram shown. The insert
probe detects the β-amyloid precursor protein mRNA
described in Figure 1 in all samples examined. The
junction probe detects the β-amyloid-related mRNA
described by Kang et al. in all cells except HeLa and
MRC5. The actin probe is a control which is expected to
hybridize to an abundant RNA in all cells.

                             6.4

                          EXAMPLE 4

                Construction of the NSE-A42 and A99

                   Transgenic Expression Plasmids

        A42 and A99 sequences were derived from the β-
actin A42 and A99 expression plasmids described in United
States patent application serial number 07/408,767.
Specifically, the β-actin A42 and the beta-actin A99
plasmids were digested with NcoI and EcoRI releasing the
β-actin promoter region as well as the SV2 neo promoter
region. In addition, by digesting with EcoRI, five amino
acids of A42 or A99 were removed which can be replaced
using a synthetic polylinker. The A42 or A99 plasmid
deleted for the β-actin and SV2 neo promoters was
purified by agarose gel electrophoresis. A synthetic
oligonucleotide polylinker which generates multiple
cloning sites as well as the five amino acids for A42 and

-47-

A99 was synthesized and ligated to the A42 or A99 plasmid
fragment generated earlier by NcoI and EcoRI digestion.
Addition of the polylinker deletes the NcoI site in the
original plasmid and moves this site to within the

5   polylinker, and replaces the EcoRI site within the A42 or
A99 sequences. After ligation of the polylinker to the
A42 or A99 plasmid fragment, plasmid DNA was prepared.
The new plasmid was next cleaved with BglII and NcoI.
Both of these sites were synthesized into the newly added

10   polylinker region. It is in the site generated by BglII-
NcoI digestion that the NSE promoter will be added.

            The NSE promoter was isolated from a rat genomic
library. An 11 kb genomic EcoRI fragment was selected
using hybridization with oligonucleotides designed from

15   the known NSE promoter region sequence. The 11 kb genomic
EcoRI NSE promoter fragment was cloned into a pUC plasmid
and it was from this genomic fragment that the desired
promoter region was isolated using PCR amplification as
outlined in Fig. 9. A large portion of the promoter

20   region containing an intron in the 5' untranslated region
of the NSE gene was produced by PCR amplification using
oligonucleotide primers with BglII and NcoI restriction
sites at the 5'- and 3' termini, respectively. The NSE
promoter fragment was purified by gel electrophoresis and

25   then cloned into the BglII-NcoI polylinker region of the
A42 or A99 plasmid generated earlier. Standard molecular
recombinant techniques were used to digest, ligate and
isolate the fragments. DNA sequencing documented the
fidelity of the NSE-A42 and NSE-A99 expression plasmids.

30

35

-48-

6.5

EXAMPLE 5

Construction of the NSE-A695 and the NSE-A751

Transgenic Expression Plasmids

5   The NSE-A99 plasmid made in Example 4 was used
to prepare NSE-A695 and NSE-A751 expression plasmids by
removing the A99 sequences and replacing them with either
A695 or A751 sequences. To remove the A99 sequences from
the NSE-A99 plasmid, the plasmid was digested with NcoI
10   followed by mung bean nuclease digestion. The mung bean
nuclease treatment generates a blunt end at the NcoI site
for further cloning purposes. The plasmid was next
digested with HindIII. This site is within the polylinker
region 3' to the A99 sequences causing the release of the
15   A99 coding fragment. The remaining plasmid containing
the NSE promoter, the plasmid sequences, the poly A
addition site, and part of the polylinker region was
purified by gel electrophoresis. Next, the A695 and the
A751 fragments were prepared for cloning into the A99-
20   deleted NSE plasmid. The A695 and the A751 sequences
were derived from β-actin A695 and β-actin A751
expression plasmids. To remove the 695 and 751
sequences, the plasmids were digested with NruI and
HindIII. The NruI site is immediately 5' to the
25   initiating methionine codon of 695 and 751. The HindIII
site is located in the polylinker region 3' to the
termination codon of 695 and 751. The NruI-HindIII
fragment containing A695 or A751 sequences was cloned
into the blunted NcoI-HindIII treated A99-deleted NSE
30   plasmid. The final plasmids, NSE-A695 and NSE-A751, were
documented as correct by DNA sequencing of the cloning
junctions. These plasmids were proven to express 695 and
751 protein after transfection into mammalian cells.

35

6.6

EXAMPLE 6

Preparation of the Metallothionein A42 and

Metallothionein A99 Transgenic Expression Plasmids

5   A synthetic metallothionein I mouse promoter
designed from the published sequence was prepared using
oligonucleotide synthesis and ligation. The synthetic
metallothionein or MT promoter was cloned into pUC19. To
prepare the MT-A99 and the MT-A42 transgenic expression
10   plasmids, the A42 and the A99 sequences were isolated
from the β-actin A42 and A99 expression plasmids,
respectively. The A42 and A99 sequences were excised
from the β-actin expression plasmid using digestion with
SalI and BamHI for A42 and SalI and HindIII for A99. The
15   SalI site common to A42 and A99 is 5' to the initiating
methionine of A42 and A99. The BamHI site and the
HindIII sites are both contained in the polylinker region
3' to the A42 and A99 coding sequences. The SalI-BamHI
fragment for A42 and the SalI-HindIII fragment for A99
20   were purified by gel electrophoresis. The synthetic MT
promoter fragment was isolated by digestion of the pUC
plasmid with EcoRI and SalI. The EcoRI-SalI MT promoter
fragment was ligated with the SalI-BamHI A42 fragment.
Similarly, the EcoRI-SalI MT promoter fragment was
25   ligated to the SalI-HindIII fragment of A99. The MT
promoter-A42 and the MT promoter-A99 fragments were then
cloned into a plasmid backbone for a complete expression
plasmid. The plasmid backbone used is the parent β-
actin vector from which the modified pAXneoR plasmid was
30   derived. The plasmid vector was prepared for cloning by
digestion with EcoRI and BamHI for insertion of the RI-
BamHI-MT-A42 fragment or the plasmid backbone was
digested with EcoRI and HindIII for insertion of the
EcoRI-HindIII-MT-A99 fragment. Standard methodologies for
35   recombinant DNA manipulation were used to create the

plasmids. The MT-A42 and MT-A99 transgenic expression
plasmids were verified as correct in sequence by DNA
sequence analysis.

5   6.7

                              EXAMPLE 7

                   Construction of MT-A751 and MT-A695
                     Transgenic Expression Plasmids

        A promoter plasmid was constructed using the
10   synthetic MT-1 fragment earlier described. This plasmid
      also contains SV40 termination region possessing an
      intron. Between the promoter and the SV40 termination
      sequences, there exists an XbaI site. The plasmid was cut
      at this XbaI site then treated with mung bean nuclease to
15   create a blunt end. It is in the blunted XbaI site of
      the MT plasmid that the 751 and 695 sequences will be
      inserted. 695 and 751 complete coding sequences were
      derived from the β-actin 695 and the β-actin 751
      expression plasmids. To obtain this fragment the β-
20   actin expression plasmids were digested with NruI and
      HindIII. The NruI site is immediately 5' to the
      initiator methionine of A695 and A751. The HindIII site
      is located 3' prime to the termination codon of A695 and
      A751 within the polylinker region of the plasmid.
25   Digestion with NruI creates a blunt terminus. Digestion
      with HindIII creates a sticky end which was filled in
      with Klenow to make a blunt ended fragment at both the 3'
      and 5' termini of 695 and 751. The 695 and the 751 blunt
      end fragment was cloned into the blunted XbaI
30   metallothionein SV40 plasmid. The resulting MT-A695 and
      MT-A751 plasmids were documented as correct in sequence
      at the cloning junctions by DNA sequence analysis.

              Figure 11A shows a schematic diagram of NSE-A751
      transgene. The 2.3 kb NSE promoter and associated
35   5'-untranslated region is indicated by the cross-hatched

box. The intron within the NSE DNA is indicated by
lines. A751 sequences are represented by the solid box.
The SV40 late region 3'-untranslated region containing
polyadenylation signals is indicated by the open box.

5   Dashed lines define plasmid sequences. Restriction
endonuclease sites, as well as probes (1-4) used for
Southern blot and reverse transcriptase PCR analyses are
indicated.

10

6.8

EXAMPLE 8
Collecting and Injecting the Eggs

6.8.a. Procedure

15   Fertilized ova were collected from the oviducts
of JU female mice previously mated with males. The ova
were collected at an early pronuclear stage wherein the
male and female pronuclei are separated and
distinguishable within the cytoplasm. The collected ova

20   were separated from any surrounding cells and materials,
properly washed and stored in accordance with procedures
known to those skilled in the art. The zygotes were
preferably stored in a depression slide containing
culture medium overlayered with paraffin oil in an

25   atmosphere of 5% carbon dioxide, 5% oxygen, and 90%
nitrogen (percentages are based on volume) at 37' C.

            A fusion construct of NSE with 5' intron
connected to A751 was obtained in accordance with the
procedure described above and shown within Figure 9. Any

30   of the above-mentioned fusion constructs can be cloned
and included within a fertilized ovum in a manner as
described herein. Accordingly, the following is a
specific description with respect to A751 but is a
generalized procedure applicable with any of the above-

35   described fusion constructs.

First, the fusion constructs were cloned as schematically shown within Figure 10. After cloning, the fusion constructs were extracted from the host, purified and then digested with the appropriate nuclease in order

5 to remove as much of the undesired bacterial sequences as possible. The extracted plasmids are purified by cesium chloride ethidium bromide density gradient centrifugation followed by extraction of the ethidium bromide and dialysis of the plasmid DNA. After carrying out such

10 extraction and purification processes on any of the cloned sequences, it is possible to obtain relatively purified plasmids containing the desired fusion constructs.

The purified plasmids were then treated with the

15 appropriate endonuclease (SalI and NdeI were used to obtain linear fragments of NSE-A751 or NSE-A695). By utilizing gel electrophoresis the desired fragments were isolated by the use of Gene Clean glass beads (sold by Bio 101, San Diego, California) followed by filtration

20 through a 0.22µ membrane.

Purified DNAs obtained were microinjected utilizing injection pipettes with an external diameter of about 1 µl. Such pipettes can be prepared from Pyrex tubing as described in Proc. Natl. Sci. U.S.A., 74:5657-

25 5661 (1971). Approximately ten picoliters of the solution containing the fusion constructs (which is approximately 20,000 DNA sequences) was drawn into an injection pipette. The zygote was positioned on a holding pipette which has an external diameter of about

30 60 to 70 microliters, which pipette can also be prepared in accordance with the procedure of the above-cited publication. The zygote was positioned on the holding pipette so that the male pronucleus was injected with the fusion constructs present in the injection pipette.

35

All of the zygotes were microinjected, then placed in culture tubes where they were allowed to develop for five days. Suitable conditions for preimplantation development are described within <u>Biol.</u>

5   <u>Reprod.</u> 8:420-426 (1973). Ova which developed to morulae or blastocytes were transplanted into the uteri of F_1 hybrid JU, foster mouse mothers who were at day 3 of pseudopregnancy. These foster mothers carried the implanted embryos to term.

10   Injection techniques of the type described above and of the type known to those skilled in the art were utilized in order to inject embryos with different conjugates of the invention as shown below within Table 1, wherein experiments 1-5 were carried out using

15   the different conjugates on different numbers of mouse embryos. The expected and actual results of these experiments are tabulated within Table 1 which shows the number of mouse eggs injected, the number of live pups which resulted from the implantation of those eggs and

20   the number of pups which actually turned out to be transgenic, that is, which actually turned out to incorporate the foreign DNA which were injected into the embryos.

25

30

35

Table 1

EXPERIMENTS

	EXPECTED	#1 NSE/695	#2 NSE/695	#3 NSE/751	#4 NSE/99	#5 NT/99
EMBRYOS INJECTED	200	289	148	233	236	288
LIVE PUPS	20-40 (10-20%)‡	7(2.4%)	7(4.7%)	44(19%)	28(12%)	25(8.7%)
TRANSGENIC ANIMALS	2-8 (10-20%)* [1-4%]**	3(43%) [1%]	1(14%) [0.6%]	9(20%) [3.8%]	3(11%) [1.3%]	3(12%) [1.0%]

‡   % of embryos resulting in live pups.

*   % of live pups who are transgenic.

** % of embryos injected resulting in live transgenic animals.

The transgenic mice obtained by experiments 1-5
as shown in Table 1 above are useful in determining the
effectiveness of a drug in decreasing the amount of
plaques formed as a result of Alzheimer's disease.

5   Transgenic mice from any one of the experiments can be
used in comparison with control mice which are
nontransgenic or various combinations of the transgenic
mice can be compared with each other and/or with the
controls with the object being determining the

10 effectiveness of one or more drugs in decreasing the
amount of plaques formed and thus relating the decrease
in the plaques with the effectiveness of the drug in
treating Alzheimer's disease.

15   EXAMPLE 9
           Determination of Transgene Copy Numbers
           For the following examples, results are
described for embryos and mice injected with NSE-A751
DNA.

20   As indicated in Table 1, 9 of 44 mice that
developed from embryos injected with NSE-A751 DNA carried
the transgene. Breeding of these mice resulted in the
establishment of 8 lines; the transgene of one founder
was not inherited by progeny. Three pedigrees were

25 selected for further characterization, including
founder 10 (F10), founder 11 (F11) and founder 24 (F24).
(Table 2). Transgene copy numbers were estimated by
comparison to the endogenous single copy b-APP mouse gene
using Southern blot hybridization with an oligonucleotide

30 probe common to both mouse b-APP and human b-A751
(Figure 11B). Forty mg of tail DNA was digested with
BglII and electrophoresed on an 0.8% agarose gel.
Southern blots were prepared and hybridized with an
oligonucleotide probe (Figure 11A, probe #1;

35

5'-GGATGTGTACTGTTTCTTCTTCA-3') radiolabeled with 32P by
T4 kinase.

Blots were hybridized at 60°C in 6 X SET (1X
SET=0.15M NaCl, 30 mM Tris-HCl pH.0, 2 mM EDTA) with 5 X
5   Denhardt's solution and washed at 608,8C for 40 minutes
in 6 X SSC (1X SSC=0.15M NaCl, 0.15M Na citrate). F10,
F11, and F24 lines had approximately, 1, 4, and 8 copies
of NSE:b-A751 per haploid genome, respectively.

10   6.8.b. Analysis

EXAMPLE 10
RNA Expression of Inherited Transgenes
RNA expression of inherited transgenes was
15   studied in three pedigrees. Two mg of total brain RNA
was isolated from positive and wild-type control animals,
reverse transcribed with oligo dT12-18, and a specific
DNA subfragment amplified by polymerase chain reaction
(PCR). The reaction was divided into 2 aliquots.
20   Oligonucleotide primers for the PCR were
designed such that only transcripts derived form the
transgene would be amplified, i.e., one primer hybridized
to the NSE 5'-untranslated region
(5'-CACCGCCACCGGCTGAGTCTGCAGTCCTCG-3') and the other the
25   5'-coding domain of the b-APP
(5'-TCTTGCACTGCTTGCGGCCCCGCTTGCACC-3'). To account for
contaminating genomic DNA or unprocessed transcripts, the
NSE PCR primer corresponded to a site located upstream
from the intron. A predicted 373 basepair fragment was
30   amplified from reverse transcribed DNA prepared from each
transgenic animal, but was not amplified from reverse
transcribed RNA isolated from wild-type mice. As a
control, the second aliquot of the reversed transcribed
RNA was amplified with a primer for native b-APP
35   secretory signal (5'-TTGGCACTGCTCCTGCTGGCCGCCTGGACG-3')

sequence and the 5'-coding domain b-APP primer described
above. In these reactions, both wild-type and transgenic
reverse transcribed samples produced the 307 basepair DNA
fragment representing amplification from endogenous b-APP
5   RNA.

DNA was electrophoresed on 2% agarose gels,
visualized by staining with ethidium bromide, and then
Southern blotted and hybridized with a 32P-labeled
oligonucleotide probe to the NSE-A751 fusion sequence
10 (5'-AGATCCCAGCCACCGATGCTGCCCGGTTTG-3').

Blots were hybridized at 65°C in 6 X SET and
washed at 65°C for 40 minutes in 4 X SSC. When the PCR
reaction products were hybridized with the
oligonucleotide probe bridging the junction between NSE
15 and b-A751 sequences, only the products derived from the
transgenic brains hybridized with the probe,
demonstrating the authenticity of the 373 bp PCR product.

EXAMPLE 11
20 Western Blot Analysis
Changes in protein expression in brains of
NSE:b-A751 transgenic animals were determined by Western
blot analysis. Protein homogenates were made from total
brain according to the methods of Shivers, B.D., et al.,
25 EMBO J (1988) 7:1365-1370. Fifty mg of each sample was
electrophoresed on a 7.5% SDS polyacrylamide gel and
transferred to membranes.   Western blots were developed
using a 1/500 dilution of polyclonal antiserum raised
against full length human A695 expressed by recombinant
30 vaccinia virus and ^{125}I-protein A. Several bands of
about 120 kD corresponding to the reported average size
of mammalian brain b-APP isoforms were observed in
controls and in each transgenic protein homogenate.
Slightly increased levels of b-APP were seen in the
35 NSE-A751 samples relative to wild-type samples suggesting

elevated levels of A751 expression in transgenic brains.
An accurate assessment of neuronal levels of exogenous
A751 expression was difficult to determine, however, due
to combined neural and glial expression of endogenous

5   b-APP. Therefore, neuronal levels of exogenous A751
expression were examined by immunocytochemistry of
NSE-A751 expression.

                          EXAMPLE 12

10   Histological Analysis of Transgenic Mouse Brains
                     with Monoclonal Antibody 4.1
               Monoclonal antibody 4.1 was used for
     histological analysis of transgenic mouse brains. This
     antibody recognizes an epitope which been mapped to the

15   N-terminal 10 residues of the β-amyloid protein and has
     high affinity and specificity for neuritic plaques. Any
     immunoreactivity was eliminated by preincubating the
     monoclonal antibody with the synthetic peptide immunogen
     prior to staining. Briefly, a synthetic peptide

20   corresponding to residues 1-28 of the β-amyloid protein
     was prepared and self-aggregated by freezing and thawing.
     The peptide aggregate was mixed with methylated bovine
     serum albumin and adjuvant for immunizing and boosting
     mice. Hybridomas from sensitized spleen cells were

25   generated. Clones secreting anti-peptide antibodies were
     expanded and subcloned by limiting dilution. Brains of
     NSE-A751 transgenic mice, wild-type mice, and homozygotic
     and hemizygotic animals from three transgenic lines were
     analyzed (Table 2). Brains were removed and mixed with

30   4% paraformaldehye, embedded in paraffin and 6 mm coronal
     midbrain sections from made. Sections were
     deparaffinized, rehydrated, treated for 30 minutes with
     0.3% H_2O_2, then with 80% formic acid for about 2 minutes.
     Sections were then incubated at 37°C for 30 minutes with

35   1/20 dilution of conditioned medium from the hybridoma

secreting 4.1 antibody. An anti-mouse avidin-
biotinylated horseradish peroxidase (ABC) kit was used
according to the manufacturer's suggestion (Vector,
Burlingame, CA), and the horseradish peroxidase visualize

5   with 3,3'-diaminobenzidine. Sections were counterstained
with hematoxylin and eosin. For staining of full-length
b-APP, a 1/400 dilution of antiserum raised against
full-length human b-APP695 expressed by recombinant
vaccina and an anti-rabbit ABC kit was used. Sections

10   were counterstained with hematoxylin and eosin. Human
brain sections were obtained from individual clinically
diagnosed with Alzheimer's disease. Human sections were
prepared and stained identically as mouse tissue sections
except they were treated with 98% formic acid for 10

15   minutes. For competition experiments, the antibody
diluent was preincubated at 4°C for 12 hours the 37°C for
30 minutes with 250 mg/ml 1-28 β-amyloid synthetic
peptide prior to application. As shown in Figure 12A and
12B, the antibody selectively stains neuritic plaques in

20   human tissue section and immuoreactivity is eliminated by
preincubating the monoclonal antibody with the synthetic
peptide immunogen prior to staining.

Transgenic and wild-type brain sections were
stained in parallel with the 4.1 antibody. Greater

25   amounts of immunoperoxidase reactivity were observed in
neurons and throughout the neuropil of transgenic brains
compared with brains from wild-type mice.

Figure 12C shows an example of increased
neuronal staining in the hippocampal pyramidal cell layer

30   from an animal of NSE-A751 founder 10 pedigree (Figure
12C). Significant staining of neuritic processes was
also observed. Enhancement of arborizing neuronal
process was most evident in stratum flanking the
pyramidal cell layer of the CA-1 and CA-3 regions of

35   transgenic hippocampi. Enhanced staining of neuritic

processes in the deeper cortical layers of transgenic brains was also seen. Neuronal and process staining was fully competed by prior incubation of the antibody with the 28 residue synthetic b-amyloid peptide and was

5   decreased by formic acid treatment. Additionally, as shown in Figure 12D, neuritic staining in transgenic brains was detected using antibodies raised to full-length b-APP indicating the immunoreactive material in neuronal process is full-length b-APP.

10   Extracellular immunoreactive deposits were also seen in brain sections from the three transgenic lines stained with the 4.1 monoclonal antibody (Figure 13A-D). These deposits are not seen in wild-type animals studied. The deposits vary in size, shape, and frequency. Compact

15   deposits of 10-30mm in diameter are shown in Figure 13A-E. Deposits were observed most frequently in the cortex and hippocampus although they occasionally were seen in the thalamus and striatum. The deposits tend to occur in clusters. On the average 5 deposits were seen

20   in a single whole brain section from NSE-b-A751 founder 10 or 11, however, fifteen 10-50mm sized deposits have been observed in a single section. Amorphous or granular immunoreactive extracellular deposits were also seen in transgenic brains section stained with the 4.1 monoclonal

25   antibody and not in control brain sections. Figure 13e shows an example of diffuse β-amyloid immunoreactivity in the hippocampus of an animal from NSE-b-A751 founder 11 line. Detection of extracellular deposits in tissue section from transgenic animals required treatment with

30   formic acid. Staining of the structures was competed by the β-amyloid peptide as seen in Figure 14e. Deposits stained variable with antibodies raised to full length b-APP. Brains from NSE-A751 founders 10 and 11 displayed a greater number of compact deposits than NSE-b-A751

35

founder 24 (Table 2) even though founder 24 has more
copies of the NSE-bA751 transgene.

Table 2
Summary of Mice Employed for Immunohistology

Line	Animal	Sex	Age[1]	Genotype	Copy #[2]	Deposits[3]
NSE-751						
10	0	F	12	Aa	1	+++
	31*	F	7	Aa		++
	168	F	5	Aa		+++
	334	M	2	AA		+
11	0	M	15	Aa	4	+++
	51	M	12	Aa		+
	236	M	4	AA		+++
	287	F	3	AA		+
24	77	M	8	Aa	8	+
	201	F	5	AA		+
Wild-type	1	F	4	n.a.	n.a	-
	2	F	4			-
	3	M	5			-
	4	M	3			-
	5	M	9			-
	6	F	12-			
	7	M	14			-

[1]month; [2]haploid; [3]>5mm in size, (-) (+/++/+++) relative
abundance of deposits, i.e., +, <5; ++, 5-10; +++, >10
deposits per section as an average of multiple sections
stained. M and F indicate male and female mice,
respectively; AA and Aa represent homozygotic and

hemizygotic animals, respectively; n.a., indicate not
applicable. ; *NSE-A751 F10 #31 died spontaneously of
unknown cause.

5   Those mice harboring exogenous genes can be
bred to 1) document that the NSE-751 or NSE-695 DNA is
stably incorporated into the genome, 2) create a pure
line, 3) characterize mRNA and protein expression, 4)
ultimately investigate possible pathological conse-
10   quences of expression of the foreign DNA, and 5) test
potential therapeutic compounds.
          A variety of analytical procedures can be
utilized to identify and characterize transgenic animals
produced in accordance with the above-described
15   protocols. It is, of course, necessary to identify which
of the transgenic animals include the inserted sequences
such as the NSE-A-99, NSE-A-42, MT-A-99 and MT-A-42.
These inserts, as well as A4i inserts, can be detected
utilizing identical rationale and specific PCR and
20   Southern blotting oligonucleotide reagents and antibodies
for Western blotting. A variety of different techniques
and combinations of techniques will become apparent to
those skilled in the art upon reading this disclosure.
          Western blotting methods to identify A751 or
25   A695 precursor in rodent brain have been developed. As an
example, a Western blot using antiserum to A695 (raised
using the recombinant vaccinia virus system) reacted with
total brain homogenate was carried out. A single protein
was recognized of 120 kd which is the predicted molecular
30   weight of the precursor. The membrane to which the
protein is transferred for the blot greatly influences
protein transfer. For proteins of greater than 30 kd,
polyvinylenedifluoride (PVDF) is optimal, whereas, below
30 kd, nitrocellulose is more efficient. Other available
35   antisera developed to regions of the amyloid precursor,

in particular, sera to the Kunitz inhibitor domain and to
the core domain may be used in Western blot analysis of
total brain protein. Additionally, by using procedures
known in the art, it is possible to isolate monoclonal
5   antibodies to the precursor. Mice have been immunized
with a recombinant vaccinia virus which expresses A751
and were shown to raise antibodies to this protein.

Figure 11B shows a Southern blot of DNA from
wild-type and transgenic mice. Lanes 1 and 2 , wild-type
10   (WT); lanes 3 and 4, NSE-A751 founder 10 (F10); lanes 5
and 6, NSE-A751 founder 11 (F11); lanes 7 and 8, NSE-A751
founder 24 (F24). The arrow indicates endogenous mouse
b-APP gene. Dots indicate exogenous b-APP.

Figure 12 (A, B, C and D) show immunoperoxidase
15   staining of human and mouse brain. Figures 12A and 12B
show human Alzheimer's disease tissue section from caudal
hippocampus stained with 4.1 antibody without
preincubation (12A) and with preincubation (12B) with the
b-amyloid synthetic peptide immunogen. Figure 12C shows
20   pyramidal cell layer of hippocampal CA-1 region of
NSE-A751 F10 (#334) stained with 4.1 antibody. Figure
12D shows the same region from wild-type mouse (#3)
stained with 4.1 antibody. Magnification is 500X.

Figure 13 (A, B, C, D and E) are
25   photomicrographs of immunoreactive deposits in NSE-A751
brains. Figure 13A is a compact deposit in frontal
parietal cortex of F11 (#0); Figure 13B is compact
deposit in thalamus of F11 (#236); Figure 13C shows
compact deposit in hippocampal CA-2 field of F11 (#0);
30   Figure 13D shows cluster of deposits in frontal parietal
cortex of F10 (#168); Figure 13E shows amorphous deposits
in the hippocampal stratum moleculare of F11 (#236).

35

7. **Uses of the Invention**

The transgenic mammals of the present invention are useful in determining the effectiveness of pharmaceutical drugs with respect to their ability to

5   decrease the amount of plaques which form within the brain of the animal. More specifically, the mammals are useful in testing the efficacy of such drugs in preventing the formation or reducing the amount of β-amyloid plaques formed as well as eliminating or

10   reducing plaques already formed.

Methods of producing the transgenic animals of the invention have been described above. Once produced, a drug to be tested is administered to a control animal or group of animals which are not the transgenic animals

15   of the invention and simultaneously to transgenic animals of the invention. The drug is preferably continuously administered over a period of time which is normally sufficient to effect the deposition of amyloid protein deposits in the brain of the animal. After administering

20   the drug for a sufficient period of time the control animal(s) along with the transgenic animal(s) are sacrificed. Examination of the brain of the animals is made. By comparing the amount of deposits within the control animal(s) to the amount of deposits within the

25   transgenic mammal(s) of the invention a determination can be made with respect to the effectiveness of the drug in controlling the relative amount of amyloid deposits.

In that the transgenic animals of the invention can be used to test the efficacy of drugs with respect to

30   preventing amyloid deposits the animals are valuable research tools with respect to allowing researchers to test the efficacy of such drugs in treating diseases associated with such amyloid deposits such as Alzheimer's disease. However, it should be pointed out that there

35   are six known instances of disease-associated amyloid

deposits in which the nature of the precursor protein for
the amyloid protein is known: for primary amyloidosis,
the source is an immunoglobulin light chain; for
secondary amyloidosis, the precursor is amyloid A
5   protein; for familial amyloid polyneuropathy and senile
cardiac amyloidosis, prealbumin also known as
transthyreitin or a variant thereof; for medullary
carcinoma of thyroid, a procalcitonin fragment; and for
hereditary cerebral hemorrhage, gamma-trace fragment
10   which has been shown to be cystatin C. (See, e.g.,
Glenner, G. New England Journal of Medicine (1980)
302:1283; Sletton, K., et al., Biochem J (1981) 195:561;
Benditt, et al., FEBS Lett (1971) 19:169; Sletton, K., et
al., Eur J Biochem (1974) 41:117; Sletton, K., et al., J
15   Exp Med (1976) 143:993). The foregoing is a partial list
and there are at least a number of additional references
with regard to procalcitonin fragment as a precursor for
the amyloid of the thyroid carcinoma. Alternatively, or
additionally, such a precursor for β-amyloid core protein
20   may be produced in the brain or elsewhere and is
specifically deposited in the brain.
        The transgenic animals and specifically
transgenic mammals of the present invention can be used
in determining the efficacy of a variety of drugs in the
25   treatment of a variety of diseases (including those
indicated above) which diseases are associated with
β-amyloid deposits in the brain. Comparative drug
testing protocols known to those skilled in the art can
be used in connection with the transgenic mammals of the
30   invention in order to test drugs.
        Intraneuronal neurofibrillary tangles are
present in other degenerative diseases as well, but the
presence of amyloid deposits both in the interneuronal
spaces (neuritic plaques) and in the surrounding
35   microvasculature (vascular plaques) seems to be

-66-

characteristic of Alzheimer's. Of these, the neuritic
plaques seem to be the most prevalent (Price, D.L., et
al., Drug Development Research (1985) 5:59-68). Plaques
are also seen in the brains of aged Down's Syndrome

5   patients who develop Alzheimer's disease. The transgenic
animals of the present invention can be used in
connection with determining the efficacy of all types of
drugs used in connection with the treatment of diseases
associated with amyloid deposits, i.e., Alzheimer's

10   Disease, (Dutch) hereditary cerebral hemorrhage with
amyloidosis, and Down's Syndrome.
          While preferred embodiments of making and using
the invention have been described, it will be appreciated
that various changes and modifications can be made

15   without departing from the invention.

8.   Deposits
          The following cultures have been deposited with
the American Type Culture Collection (ATCC), Rockville,

20   MD, USA for patent purposes. Bacteriophage phages λSM2,
λSM2W9, and λAPCP168i4 and plasmid pNSE-β-APP751 were
deposited under the conditions specified by the Budapest
Treaty on the International Recognition of the Deposit of
Microorganisms (Budapest Treaty).

25

Culture	Accession No.	Deposit Date
λSM2	40279	13 November 1986
SM2W4	40299	29 December 1986
SM2W3	40300	29 December 1986
λSM2W9	40304	29 January 1987
λACPC168i4	40347	1 July 1987
pNSE-β-APP751	75012	22 May 1991

30

          Availability of the deposited strains is not to be

35   construed as a license to practice the invention in

contravention of the rights granted under the authority
of any government in accordance with its patent laws.

5

10

15

20

25

30

35

Claims

What is claimed is:

5

        1.   A transgenic non-human mammal whose cells
contain the cloned recombinant or synthetic DNA sequence
which sequence comprises:
        a tissue specific promoter sequence; and
10   a sequence which encodes for the production of
a β-amyloid precursor protein.

        2.   The transgenic non-human mammal as claimed
in claim 1, wherein the promoter is a nerve tissue
15 specific promoter.

        3.   The transgenic non-human mammal as claimed
in claim 2, wherein the nerve tissue specific promoter is
neuronal specific enolase (NSE).
20

        4.   The transgenic non-human mammal as claimed
in claim 2, wherein the sequence which encodes for the
production of a β-amyloid precursor protein is a sequence
which encodes for a protein selected from the group
25 consisting of: A42, A99, A695, A751 and A770.

        5.   The transgenic non-human mammal as claimed
in claim 1, wherein the sequence which encodes for the
production of a β-amyloid precursor protein is a sequence
30 which encodes for A4i.

        6.   The transgenic non-human mammal as claimed
in claim 1, wherein the mammal is a mouse.

35

7. A transgenic non-human mammal whose cells contain a cloned recombinant or synthetic DNA sequence, which sequence comprises:

a tissue specific promoter sequence; and

5   a sequence which expresses a protein having the amino acid sequence:

```
                                        10
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His
                     20
Gln  Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
    30                                      40      (42)
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala
                         50
Thr Val Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln
        60                                          70
Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala Ala
                                     80
Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn
                     90
Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln
99
Asn.
```

20   8. The transgenic non-human mammal as claimed in claim 7, wherein the tissue-specific promoter sequence is neuronal-specific enolase.

25   9. A transgenic non-human mammal whose cells contain a cloned recombinant or synthetic DNA sequence, which sequence comprises:

a tissue specific promoter sequence; and

a sequence which expresses and results in a biologically active, refolded protein having the amino

30   acid sequence:

```
                                        10
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His
                     20
Gln  Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
    30                                      40      (42)
```

35   Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala

```
                                       50
  Thr Val Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln
                60                                        70
  Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala Ala
                                          80
  Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn
5                            90
  Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln
  99
  Asn.
```

10.   The transgenic non-human mammal as claimed
10   in claim 9, wherein the tissue-specific promoter sequence
is neuronal-specific enolase.

11.   A transgenic non-human mammal whose cells
contain a cloned recombinant or synthetic DNA sequence,
15   which sequence comprises:
          a tissue-specific promoter sequence; and
          a sequence which expresses a protein having the
amino acid sequence:

```
                                       10
  Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His
                             20
  Gln  Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys

     30                                    40      (42)
  Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala.
```

25
12.   The transgenic non-human mammal as claimed
in claim 11, wherein the tissue-specific promoter
sequence is neuronal specific enolase.

30

35

13. A transgenic non-human mammal whose cells
contain a cloned recombinant or synthetic DNA sequence,
which sequence comprises:

a tissue-specific promoter sequence; and

5   a sequence which expresses and results in a
biologically active, refolded protein having the amino
acid sequence:

10
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His
20
10 Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys

30   40   (42)
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala

14. The transgenic non-human mammal as claimed
15 in claim 13, wherein the tissue-specific promoter
sequence is neuronal specific enolase.

15. A transgenic non-human mammal whose cells
contain a subfragment of the DNA sequence of Figure 1,
20 wherein the subfragment corresponds to the 168 basepair
insert fragment of the β-amyloid-related gene of
bacteriophage λAPCP16814.

16. The transgenic non-human mammal of
25 claim 15, wherein the 168 basepair insert fragment
expresses the β-amyloid precursor protein which has the
amino acid sequence:

GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet
IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla
30 ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe
AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle.

17. The transgenic non-human mammal as claimed
in claim 16, wherein the mammal is a mouse.
35

18.   The transgenic non-human mammal as claimed
in claim 15, wherein the cloned recombinant or synthetic
DNA sequence ubiquitously over-expresses A4i.

5

19.   A transgenic non-human mammal whose cells
contain a cloned recombinant or synthetic DNA sequence,
which sequence comprises:
        a tissue specific promoter sequence; and
10   a sequence which expresses a protease inhibitor
having the amino acid sequence:
        GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet
        IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla
        ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe
15   AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle.

20.   A transgenic non-human mammal whose cells
contain a cloned recombinant or synthetic DNA sequence
which expresses and results in a biologically active,
20 refolded protease inhibitor having the amino acid
sequence:
        GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet
        IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla
        ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe
25   AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle.

21.   A transgenic non-human mammal whose cells
contain a cloned recombinant or synthetic DNA sequence
which expresses an analog of human amyloid plaque core
·30 protein protease inhibitor wherein the amino acid
corresponding to arginine at position 13 in the sequence
        GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet
        IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla
        ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe
35   AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle

is substituted with an aromatic amino acid, said analog exhibiting chymotrypsin inhibitory activity.

22. The transgenic non-human mammal as claimed in claim 21, wherein the aromatic amino acid is selected from the group consisting of phenylalanine, tyrosine and tryptophan.

23. A transgenic non-human mammal whose cells contain a cloned recombinant or synthetic DNA sequence which expresses an analog of human amyloid plaque core protein protease inhibitor wherein the amino acid corresponding to arginine at position 13 in the sequence:

GluValCysSerGluGlnAlaGluThrGlyProCysArgAlaMet
IleSerArgTrpTyrPheAspValThrGluGlyLysCysAla
ProPhePheTyrGlyGlyCysGlyGlyAsnArgAsnAsnPhe
AspThrGluGluTyrCysMetAlaValCysGlySerAlaIle

is substituted with neutral hydrophobic amino acid, said analog exhibiting human elastase inhibitory activity.

24. The transgenic non-human mammal as claimed in claim 23, wherein the neutral hydrophobic amino acid is selected from the group consisting of leucine, methionine and valine.

25. A transgenic non-human mammal characterized as having a plurality of nerve tissue cells containing a nerve tissue specific promoter sequence and a 168 basepair insert fragment of the β-amyloid precursor gene of bacteriophage λPAPCP168i4 and a control sequence operably associated therewith, which, under predetermined conditions, expresses the gene under the control of the control sequence in the cell of the mammal.

26. The transgenic non-human mammal as claimed in claim 25, wherein the mammal overexpresses the β-amyloid precursor gene in neurons.

5   27. The transgenic non-human mammal as claimed in claim 1, wherein the mammal is a mouse.

28. A method of producing a transgenic non-human mammal characterized as having a plurality of cells

10   containing a cloned recombinant or synthetic DNA sequence, the sequence comprising: a nerve tissue specific control sequence operably associated with a sequence which encodes for a β-amyloid precursor protein, the method comprising the steps of:

15   (a) introducing the control sequence and associated sequence into a pronucleus of a mammalian zygote by microinjection, said zygote being capable of development into a mammal, thereby obtaining a genetically transformed zygote;

20   (b) transplanting an embryo derived from the genetically transformed zygote into a pseudo pregnant female capable of bearing the embryo to term; and

(c) allowing the embryo to develop to term; where said control sequence and associated sequence are

25   selected so that the associated sequence is not activated in such manner and degree as would prevent normal development of the embryo to term.

29. The method of claim 28 wherein the control

30   sequence is one naturally associated with the gene.

30. The method of claim 28 wherein the control sequence is one which will activate the gene when the cell is exposed to stimulus different from the natural

35   stimulus of the gene.

31. The method of claim 28 in which at least
about 1,000 copies of the gene are microinjected into the
pronucleus and the volume of genetic material injected
5   does not exceed about 10 picoliters.

32. The method of claim 28 in which the control
sequence and associated sequence is introduced into the
male pronucleus of an egg having a separate female and
10 male pronucleus.

33. The method of claim 28 in which the zygote
is allowed to develop in vitro to the morulā or
blastocyst stage prior to transplantation.
15

34. A transgenic non-human mammal characterized
as having a plurality of cells containing the cloned
recombinant or synthetic DNA sequence and a promoter
sequence operably associated therewith, which expresses
20 the DNA sequence under the control of the promoter
sequence in cells of the mammal wherein the promoter and
DNA sequence are selected from the group consisting of:

Promoter	A4 Sequence
NSE	A42 core domain
NSE	A99 carboxy tail
NSE	A751
NSE	A695
NSE	A4i
MT	A751
MT	A695
MT	A42 core domain
MT	A99 carboxy tail.
MT	A4i

35. A method of producing a transgenic non-
human mammal characterized as having a plurality of cells
containing a cloned recombinant or synthetic DNA sequence
35 comprised of an A4 sequence and a promoter sequence

operably associated therewith, which, under predetermined
conditions, expresses the A4 sequence under the control
of the promoter sequence in the cells of the mammal,
comprising the steps of:

5   (a) introducing the DNA sequence into a
pronucleus of a mammalian zygote by microinjection, the
zygote being capable of development into a mammal,
thereby obtaining a genetically transformed zygote;

           (b) transplanting an embryo derived from the
10   genetically transformed zygote into a pseudo pregnant
female capable of bearing the embryo to term; and

           (c) allowing the embryo to develop to term;
where said A4 sequence and promoter sequence are selected
from the group consisting of:

15

Promoter	A4 Sequence
NSE	A42 core domain
NSE	A99 carboxy tail
NSE	A751
NSE	A695
NSE	A4i
MT	A751
MT	A695
MT	A42 core domain
MT	A99 carboxy tail.
MT	A4i

20

25

           36.   The method of claim 35 wherein the promoter
sequence is NSE and the A4 sequence is A751.

           37.   The method of claim 35 wherein the promoter
.30   sequence is NSE and the A4 sequence is A695.

           38.   The method of claim 35 in which at least
about 1,000 copies of the DNA sequence are microinjected
into the pronucleus and the volume of genetic material
35   injected does not exceed about 10 picoliters.

39. The method of claim 35 in which the DNA sequence is introduced into the male pronucleus of an egg having a separate female and male pronucleus.

40. The method of claim 35 in which the zygote is allowed to develop in vitro to the morula or blastocyst stage prior to transplantation.

41. A method of determining the effectiveness of a drug in decreasing amounts of plaques, comprising:

administering the drug to a transgenic mammal as claimed in claim 1 and continuing to administer the drug over a period of time normally sufficient to effect amyloid protein deposition in the brain of the mammal; and

assaying the brain for inducer associated amyloid deposits and determining the effectiveness of the drug based on the relative amount of amyloid deposits as compared to a standard.

42. The method as claimed in claim 41, wherein the standard is a standard obtained from a transgenic mammal as claimed in claim 1 which has not had the drug administered thereto.

43. A method of determining the effectiveness of a drug, comprising:

administering the drug to a transgenic mammal as claimed in claim 14 wherein the administering includes periodically providing the drug to the mammal in sufficient amounts over a period of time which would normally effect amyloid protein deposition in the brain of the mammal; and

examining the brain of the mammal for amyloid deposits and determining the effectiveness of the drug based on the relative amount of amyloid deposits as compared to a standard.

5

44. The method as claimed in claim 43, wherein the standard is the brain of a mammal as claimed in claim 14 wherein the mammal has not had the drug administered thereto.

10

15

20

25

30

35

1/16

```
                                                              ATG CTG CCC
                                                              MET Leu Pro

GGT TTG GCA CTG CTC CTG CTG GCC GCC TGG ACG GCT CGG GCG CTG GAG GTA CCC
Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg Ala Leu Glu Val Pro
                            10                                        20

ACT GAT GGT AAT GCT GGC CTG CTG GCT GAA CCC CAG ATT GCC ATG TTC TGT GGC
Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro Gln Ile Ala MET Phe Cys Gly
                              30

AGA CTG AAC ATG CAC ATG AAT GTC CAG AAT GGG AAG TGG GAT TCA GAT CCA TCA
Arg Leu Asn MET His MET Asn Val Gln Asn Gly Lys Trp Asp Ser Asp Pro Ser
40                                            50
GGG ACC AAA ACC TGC ATT GAT ACC AAG GAA GGC ATC CTG CAG TAT TGC CAA GAA
Gly Thr Lys Thr Cys Ile Asp Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu
            60                                        70

GTC TAC CCT GAA CTG CAG ATC ACC AAT GTG GTA GAA GCC AAC CAA CCA GTG ACC
Val Tyr Pro Glu Leu Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr
                        80                                    90

ATC CAG AAC TGG TGC AAG CGG GGC CGC AAG CAG TGC AAG ACC CAT CCC CAC TTT
Ile Gln Asn Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe
                            100                                    110

GTG ATT CCC TAC CGC TGC TTA GTT GGT GAG TTT GTA AGT GAT GCC CTT CTC GTT
Val Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu Val
                              120

CCT GAC AAG TGC AAA TTC TTA CAC CAG GAG AGG ATG GAT GTT TGC GAA ACT CAT
Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg MET Asp Val Cys Glu Thr His
130                                           140

CTT CAC TGG CAC ACC GTC GCC AAA GAG ACA TGC AGT GAG AAG AGT ACC AAC TTG
Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu Lys Ser Thr Asn Leu
            150                                       160

CAT GAC TAC GGC ATG TTG CTG CCC TGC GGA ATT GAC AAG TTC CGA GGG GTA GAG
His Asp Tyr Gly MET Leu Leu Pro Cys Gly Ile Asp Lys Phe Arg Gly Val Glu
                  170                                       180

TTT GTG TGT TGC CCA CTG GCT GAA GAA AGT GAC AAT GTG GAT TCT GCT GAT GCG
Phe Val Cys Cys Pro Leu Ala Glu Glu Ser Asp Asn Val Asp Ser Ala Asp Ala
                      190                                       200
```

FIG. 1-1

2/16

```
GAG GAG GAT GAC TCG GAT GTC TGG TGG GGC GGA GCA GAC ACA GAC TAT GCA GAT
Glu Glu Asp Asp Ser Asp Val Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp
                                210

GGG AGT GAA GAC AAA GTA GTA GAA GTA GCA GAG GAG GAA GAA GTG GCT GAG GTG
Gly Ser Glu Asp Lys Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val
220                                      230

GAA GAA GAA GAA GCC GAT GAT GAC GAG GAC GAT GAG GAT GGT GAT GAG GTA GAG
Glu Glu Glu Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu
            240                                  250

GAA GAG GCT GAG GAA CCC TAC GAA GAA GCC ACA GAG AGA ACC ACC AGC ATT GCC
Glu Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile Ala
                    260                              270

ACC ACC ACC ACC ACC ACC ACA GAG TCT GTG GAA GAG GTG GTT CGA GAG GTG TGC
Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg Glu Val Cys
                        280                              290

TCT GAA CAA GCC GAG ACG GGG CCG TGC CGA GCA ATG ATC TCC CGC TGG TAC TTT
Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala MET Ile Ser Arg Trp Tyr Phe
                            300

GAT GTG ACT GAA GGG AAG TGT GCC CCA TTC TTT TAC GGC GGA TGT GGC GGC AAC
Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn
310                                  320

CGG AAC AAC TTT GAC ACA GAA GAG TAC TGC ATG GCC GTG TGT GGC AGC GCC ATT
Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys MET Ala Val Cys Gly Ser Ala Ile
        330                                  340

CCT ACA ACA GCA GCC AGT ACC CCT GAT GCC GTT GAC AAG TAT CTC GAG ACA CCT
Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro
                    350                              360

GGG GAT GAG AAT GAA CAT GCC CAT TTC CAG AAA GCC AAA GAG AGG CTT GAG GCC
Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala
                        370                              380

AAG CAC CGA GAG AGA ATG TCC CAG GTC ATG AGA GAA TGG GAA GAG GCA GAA CGT
Lys His Arg Glu Arg MET Ser Gln Val MET Arg Glu Trp Glu Glu Ala Glu Arg
                        390

CAA GCA AAG AAC TTG CCT AAA GCT GAT AAG AAG GCA GTT ATC CAG CAT TTC CAG
Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile Gln His Phe Gln
400                                      410

GAG AAA GTG GAA TCT TTG GAA CAG GAA GCA GCC AAC GAG AGA CAG CAG CTG GTG
Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val
            420                                  430
```

FIG. 1-2

3/16

GAG ACA CAC ATG GCC AGA GTG GAA GCC ATG CTC AAT GAC CGC CGC CGC CTG GCC
Glu Thr His MET Ala Arg Val Glu Ala MET Leu Asn Asp Arg Arg Arg Leu Ala
            440   450

CTG GAG AAC TAC ATC ACC GCT CTG CAG GCT GTT CCT CCT CGG CCT CGT CAC GTG
Leu Glu Asn Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val
                    460   470

TTC AAT ATG CTA AAG AAG TAT GTC CGC GCA GAA CAG AAG GAC AGA CAG CAC ACC
Phe Asn MET Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr
                            480

CTA AAG CAT TTC GAG CAT GTG CGC ATG GTG GAT CCC AAG AAA GCC GCT CAG ATC
Leu Lys His Phe Glu His Val Arg MET Val Asp Pro Lys Lys Ala Ala Gln Ile
490   500

CGG TCC CAG GTT ATG ACA CAC CTC CGT GTG ATT TAT GAG CGC ATG AAT CAG TCT
Arg Ser Gln Val MET Thr His Leu Arg Val Ile Tyr Glu Arg MET Asn Gln Ser
        510   520

CTC TCC CTG CTC TAC AAC GTG CCT GCA GTG GCC GAG GAG ATT CAG GAT GAA GTT
Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp Glu Val
                530   540

GAT GAG CTG CTT CAG AAA GAG CAA AAC TAT TCA GAT GAC GTC TTG GCC AAC ATG
Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val Leu Ala Asn MET
                        550   560

ATT AGT GAA CCA AGG ATC AGT TAC GGA AAC GAT GCT CTC ATG CCA TCT TTG ACC
Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala Leu MET Pro Ser Leu Thr
                                570

GAA ACG AAA ACC ACC GTG GAG CTC CTT CCC GTG AAT GGA GAG TTC AGC CTG GAC
Glu Thr Lys Thr Thr Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp
580   590

GAT CTC CAG CCG TGG CAT TCT TTT GGG GCT GAC TCT GTG CCA GCC AAC ACA GAA
Asp Leu Gln Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu
            600   610

AAC GAA GTT GAG CCT GTT GAT GCC CGC CCT GCT GCC GAC CGA GGA CTG ACC ACT
Asn Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr
                    620   630

CGA CCA GGT TCT GGG TTG ACA AAT ATC AAG ACG GAG GAG ATC TCT GAA GTG AAG
Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys
                            640   650

ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG
MET Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu
                                660

FIG. 1-3

```
GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA GGT GCA ATC ATT GGA CTC ATG
Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu MET
670                                         680

GTG GGC GGT GTT GTC ATA GCG ACA GTG ATC GTC ATC ACC TTG GTG ATG CTG AAG
Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val MET Leu Lys
        690                                         700

AAG AAA CAG TAC ACA TCC ATT CAT CAT GGT GTG GTG GAG GTT GAC GCC GCT GTC
Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp Ala Ala Val
                710                                         720

ACC CCA GAG GAG CGC CAC CTG TCC AAG ATG CAG CAG AAC GGC TAC GAA AAT CCA
Thr Pro Glu Glu Arg His Leu Ser Lys MET Gln Gln Asn Gly Tyr Glu Asn Pro
                        730                                         740

ACC TAC AAG TTC TTT GAG CAG ATG CAG AAC TAG
Thr Tyr Lys Phe Phe Glu Gln MET Gln Asn
                        750
```

FIG. 1- 4

5/16

```
                                          10
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln
                20                                         30
Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala
                                     40      (42)
Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile
                50                                         60
Val Ile Thr Leu Val Met Leu Lys Lys Gln Tyr Thr Ser Ile
                                     70
His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu
                80                                         90
Arg His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr
                                 (99)
Tyr Lys Phe Phe Glu Gln Met Gln Asn.
```
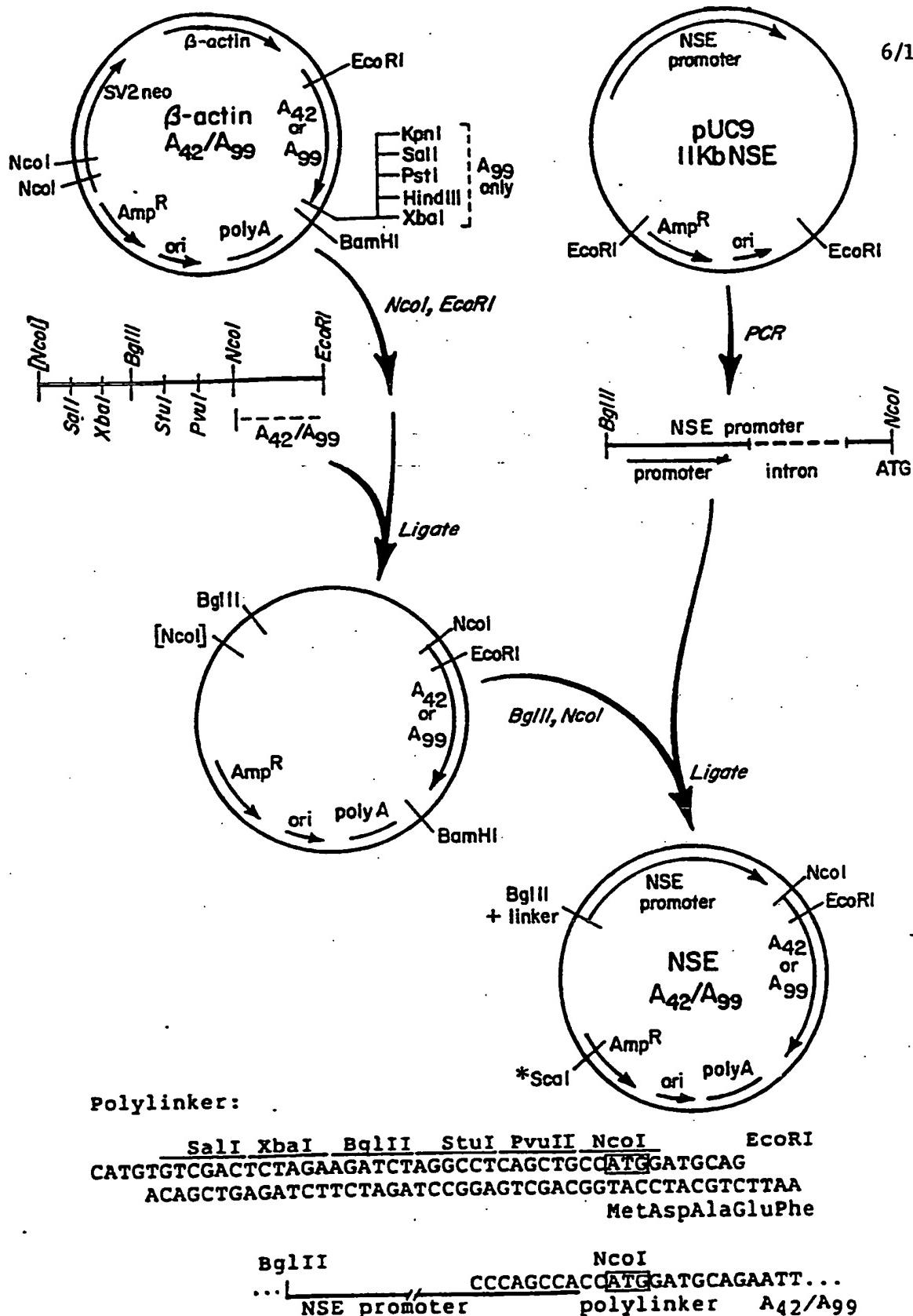
FIG. 2

```
                                          10
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln
                20                                         30
Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala
                                     40      (42)
Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala
```
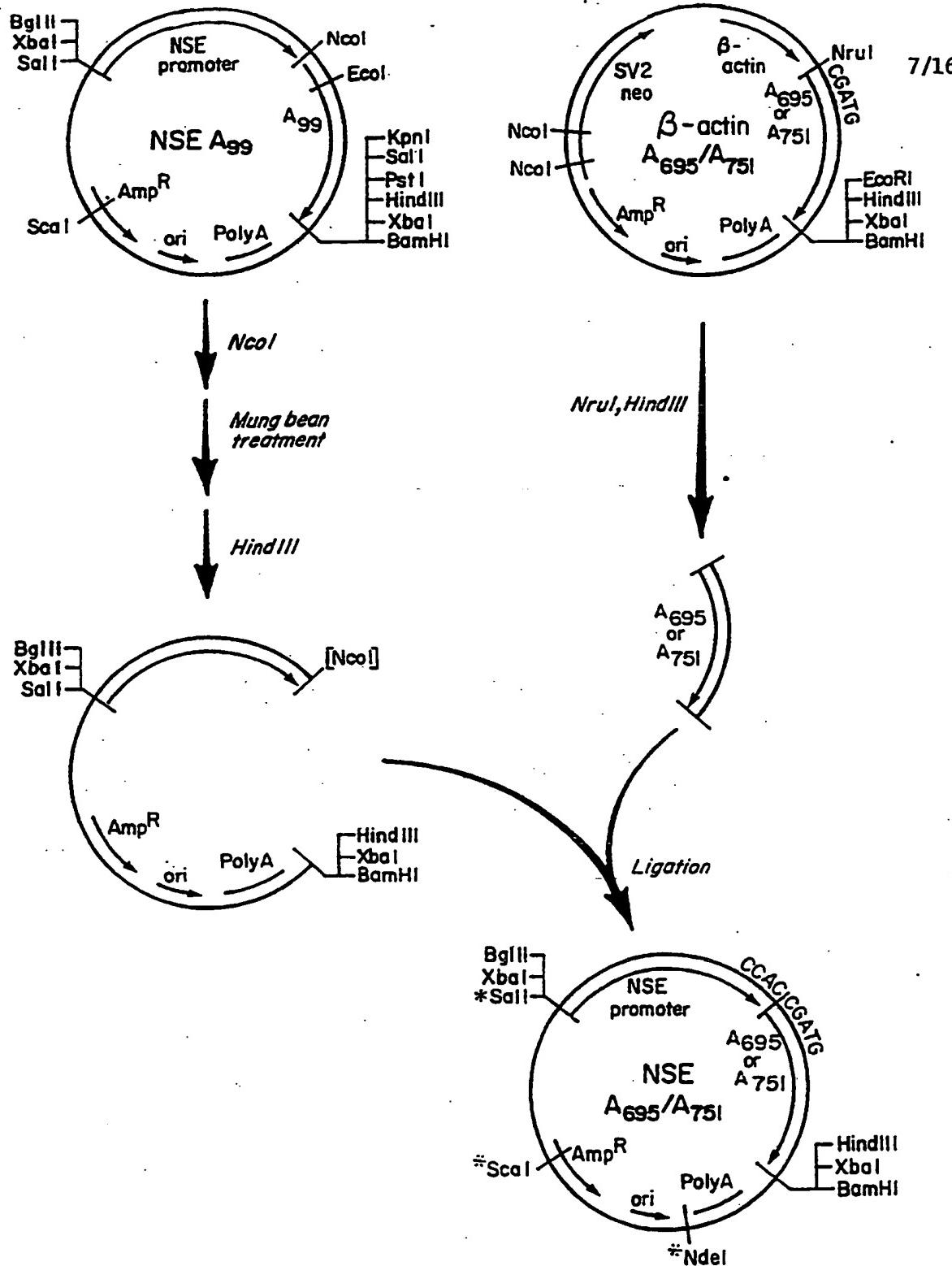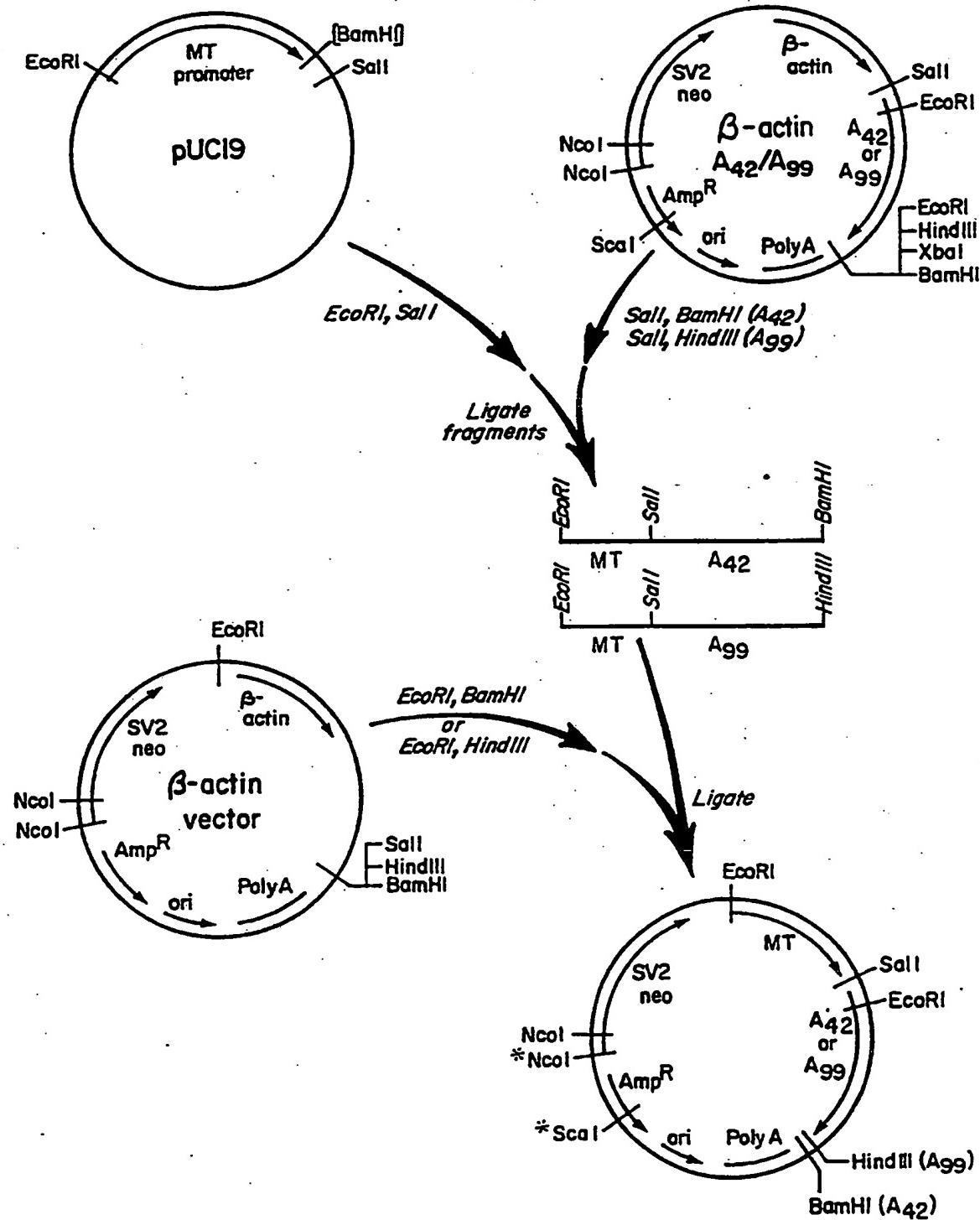
FIG. 3

Polylinker:

```
        SalI   XbaI   BglII   StuI  PvuII  NcoI            EcoRI
CATGTGTCGACTCTAGAAGATCTAGGCCTCAGCTGCC[ATG]GATGCAG
     ACAGCTGAGATCTTCTAGATCCGGAGTCGACGG[TACC]TACGTCTTAA
                                        MetAspAlaGluPhe


      BglII                              NcoI
   ...|                          CCCAGCCACC[ATG]GATGCAGAATT...
          NSE promoter                  polylinker    A₄₂/A₉₉
```

Fig. 4

Fig. 5

Fig. 6
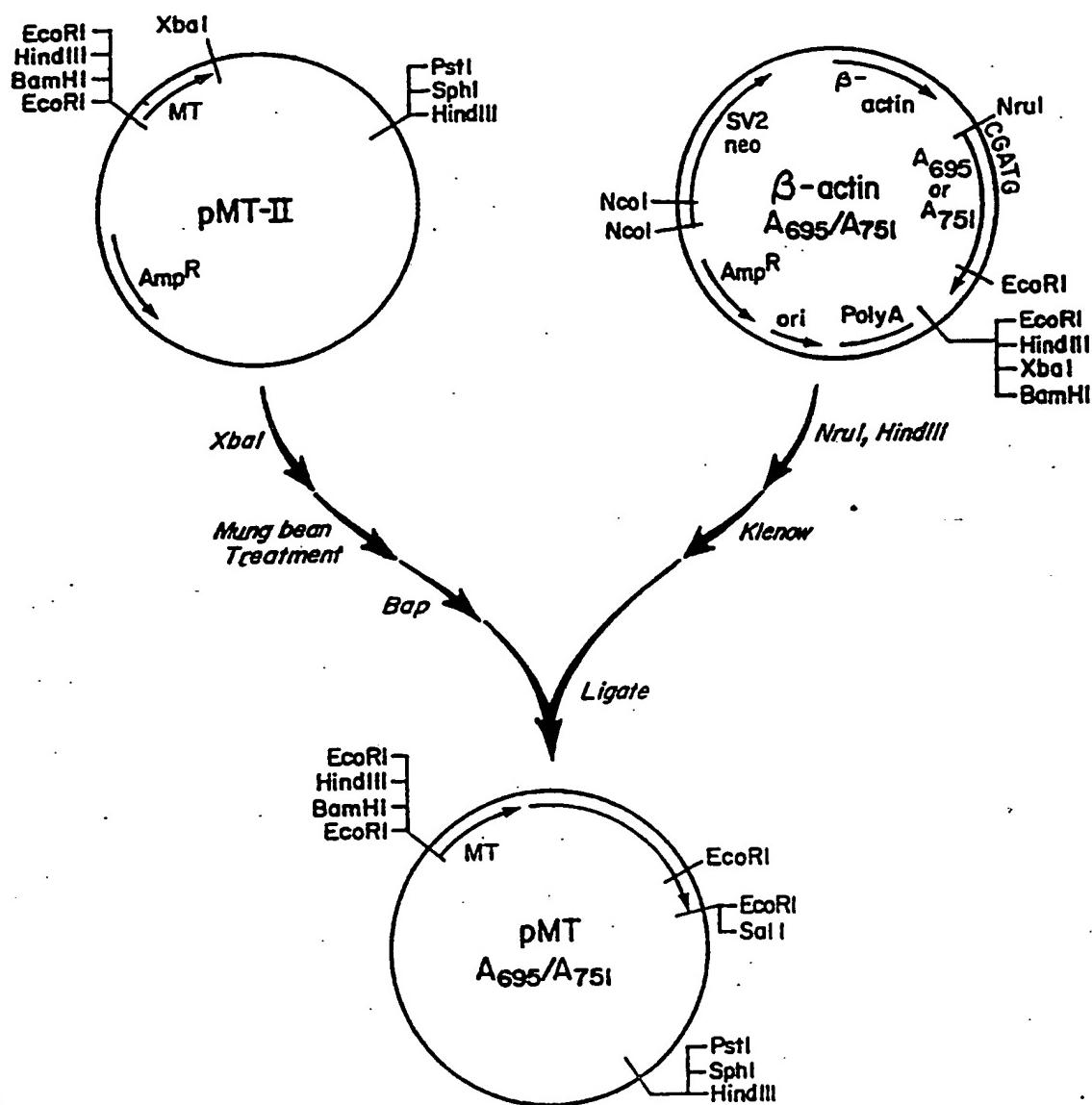
Sequence around <u>ATG</u>:

MT promoter   CCAGATCTGGA<u>ATG</u>

Fig. 7

FIG. 8-1

TIHUBI : Inter-alpha-trypsin inhibitor (BPI type)
50.0% identity in 52 aa overlap

```
        1' AVLPQEERGSGGCGQLVTEVTKKEDSCQLGYSAGPCMGMTSRYFYNGTSMACETPQYGGCM

        1'                      EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRRN
                                :.  .: :::::.: :  : :: .:::.: :  :::: :: :
       61' GNGNNFVTEKECLQTCRTVAACNLPIVIRGPCRAFIQLWAFDAVKGKCVLFPYGGCQGNGM
```

```
       42' NFDTEEYCMAVCGSAI
           .: .: : :.. ::
      121' RFYSEKECREYCGVPGDEDEELL
```

TIBOBI : Inter-alpha-trypsin inhibitor (BPI type)
48.1% identity in 54 aa overlap

```
        1'                                                           EV
                                                                     :.
        1' KADSCQLDYSQGPCLGLFKRYFYNGTSMACETPLYGGCMGNLNNFLSQKECLQTCRTVEA

        3' CSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCMAVCGSAI
           :::::.: :  : :: .:::.: ::: :: :.: .:: :: :
       61' CNLPIVQGPCRAFIQLWAFDAVKGKCVRFSYGGCKGNGNKFYSQKECKEYCGIPGEADER
```

```
      121' LL
```

FIG. 8-2

TIBO : Basic protease inhibitor precursor - Bovine
47.4% identity in 57 aa overlap

```
INSERT    1'                  EVCSEQAETGPCRAMISRWYFDVTEGKCAPFYYGGCGGNRNNFD
                                .:.:  ::::.:..: :...::  :...: :::: ..::::.
TIBO      1* PSLFMDPPIPAAQRPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFK

          45' TEEYCMAVCGSAI
               ..: :: .::.::
          61* SAEDCMRTCGGAIGPWGKTGGRAEGEGKG
```

TIBOR : Serum basic protease inhibitor - Bovine
42.9% identity in 56 aa overlap

```
INSERT    1'       EVCSEQAETGPCRAMISRWYFDVTEGKCAPFYYGGCGGNRNNFDTEEYCMAVCGSA
                    .:.: ::::.: .:...::: :..: :::: ...:::...: :: .::.:
TIBOR     1* TERPDFCLEPPYTGPCLAMIRYFYNARAGFCETFVYGGCRAKSHNFKSAEDCMRTCGGA

          57' I
```

γ NSE plus intron oligos:

BglII
5'GAA GAT CTT CAG TAA AGG TGA TGG CAC GAAGGC 3'

NcoI
TCT GTATAG CCATGG TGG CTG GGATCT CCTTGGG 3'
5'

BglII
γ NSE Pr   NcoI   11 kb genomic fragment

↓ PCR

↓ cut NcoI

BglII   NcoI

↓ subclone
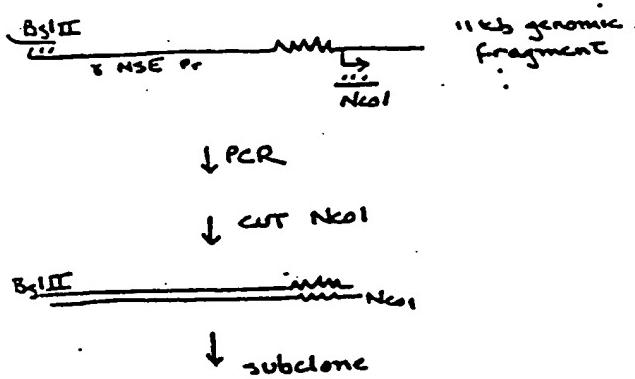
FIG. 9
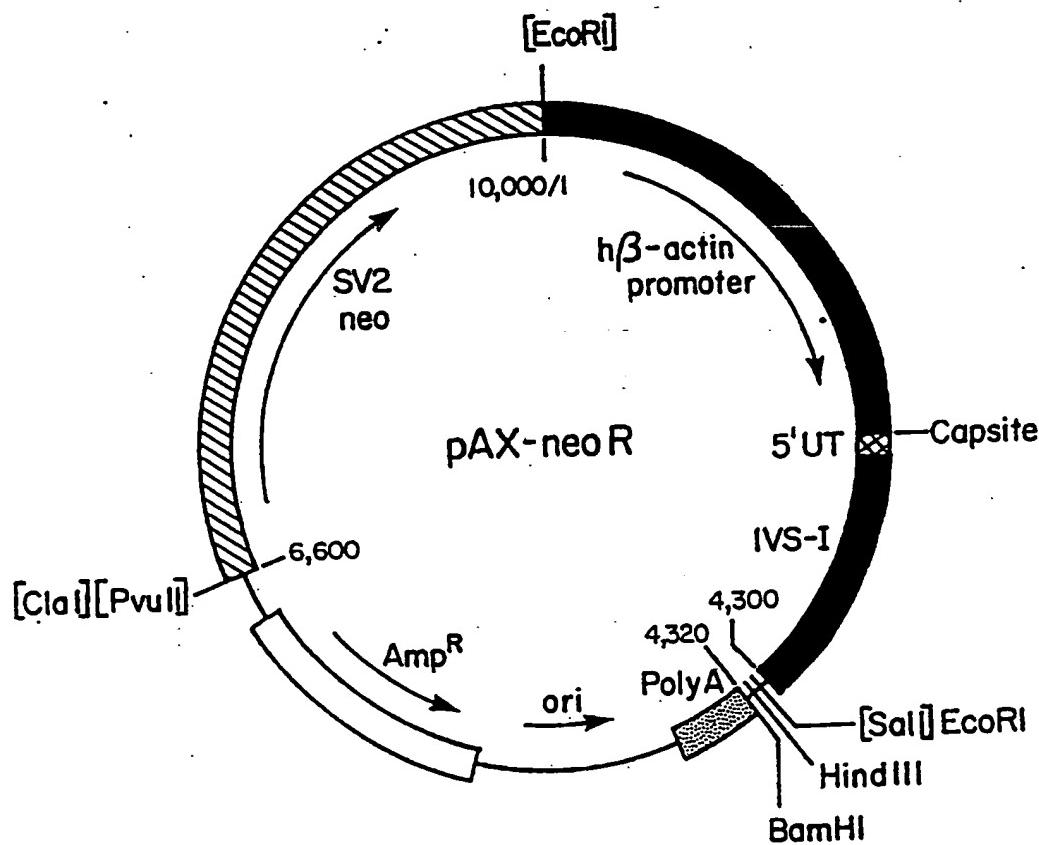
Fig.10
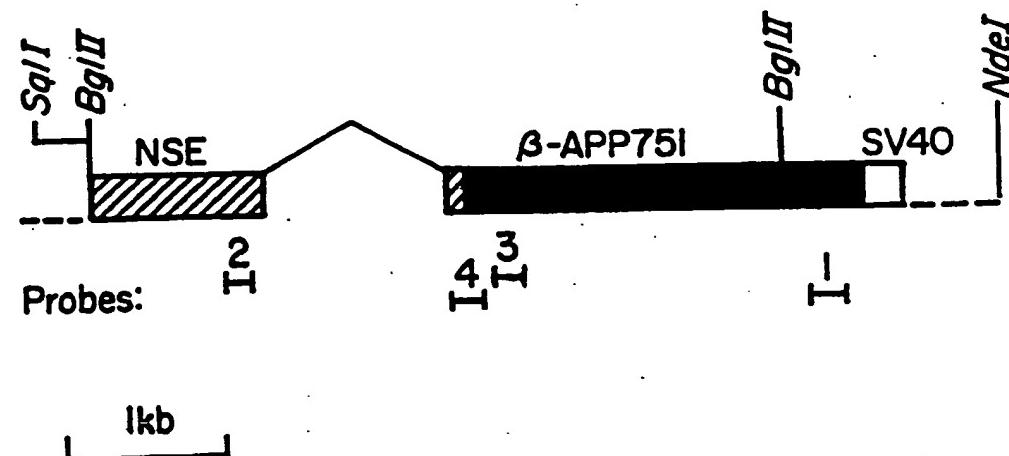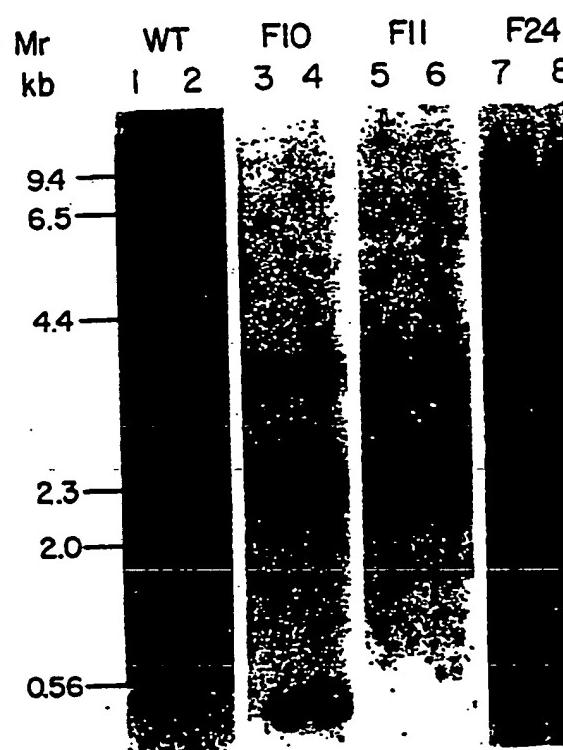
FIG. 11
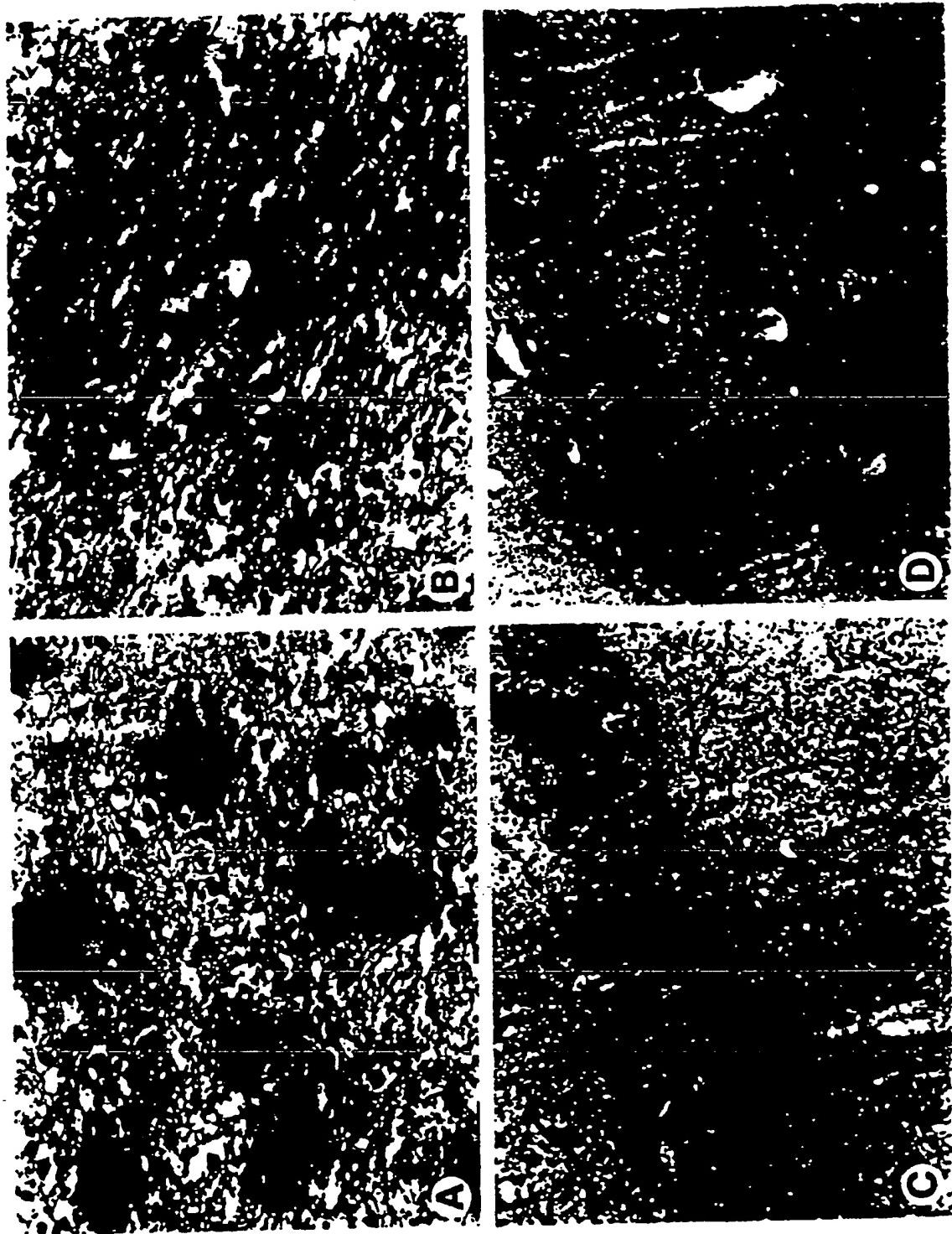
FIG. 12
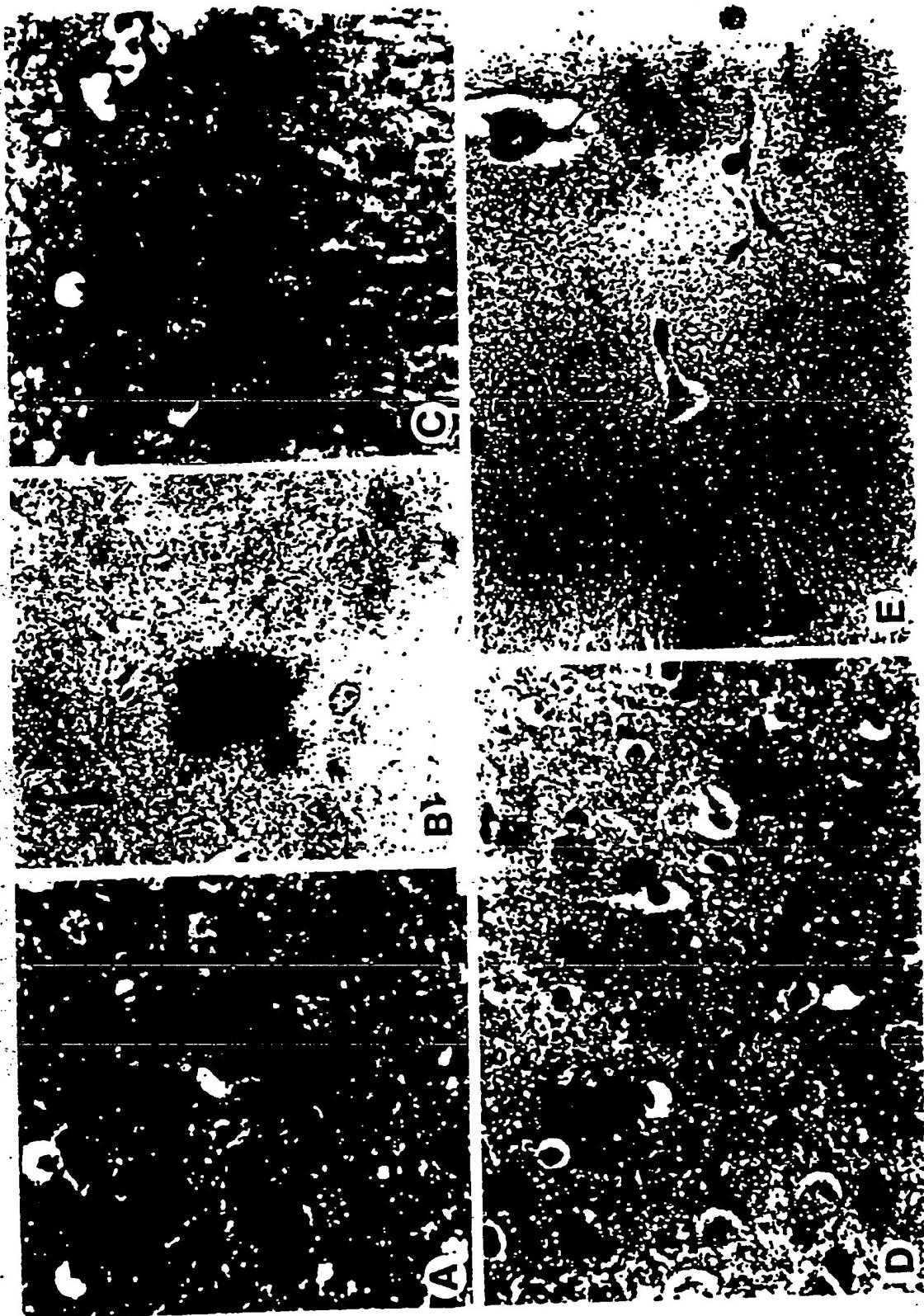
FIG. 13

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) :C12P 21/00; A01H 5/00
US CL : 800/2; 435/69.1

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	800/2; 435/69.1; 935/6

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched[5]

APS, CHEM. ABSTRACTS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category[*]	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y/A	Nature, Vol 325, Issued 19 February 1987, Kang et al, " The Precursor of Alzheimer's Disease Amlyoid A4...Receptor", pages 733-736, see entire document.	1-22/23-44
Y/A	Nature, vol 331, issued 11 February 1988, Ponte et al, "A new A4 amyloid mRNA contains... inhibitors", pages 525-527, see entire document.	1-22/23-44
Y/A	Nature, vol 317, issued 26 September 1985, Swanson et al, "Novel developmental specificity in the nervous system...genes", pages 363-366, see entire document.	1-22/23-44
Y/A	Nature, vol 331, issued 11 February 1988, Kitaguchi et al, "Novel precursor of Alzheimer's disease...activity", pages 530-532, see entire document.	1-22/23-44
Y/A	Proc. Natl. Acad. Sci., Vol 82, issued June 1985, Masters et al, "Amyloid plaque core protein...syndrome", pages 4245-4249, see entire document.	1-22/23-44

* Special categories of cited documents:[15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
27 November 1991	29 NOV 1991
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Deborah Crouch